ACCELERATED MICROBIAL DEGRADATION OF CARBOFURAN IN SOIL

By

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concerns, the potential effects of these carbofuran treatments on the soil microbial community structure were assessed using a terminal restriction fragment length polymorphism analysis. Each soil and treatment group were analyzed ten months after the last treatment with analytical carbofuran. No significant changes were observed between treatment groups of each soil. Finally, to explore the potential of molecular methods to rapidly predict the development of accelerated microbial degradation, the presence of the *mcd* gene was determined in 28 soils from five continents with and without treatment histories of carbamate pesticides using polymerase chain reaction. The *mcd* gene codes for carbofuran hydrolase. In addition, carbofuran inactivation rates of these soils were determined using ^{14}C-carbonyl carbofuran. Accelerated degradation rates were observed in 64% of the soils, and the *mcd* gene was detected in 36% of the soils. Of the soils where the *mcd* gene was detected, all but one showed accelerated degradation rates. The absence of the *mcd* gene in the remaining soils with accelerated degradation rates suggests that other mechanisms are responsible for the loss of carbofuran efficacy.

ABSTRACT

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Accelerated microbial degradation is an agricultural problem that arises when plant protectants are repeatedly applied to the same soil resulting in reduced efficacy or failure of the compound. The purpose of my doctoral research was to study accelerated microbial degradation in soils using the soil-incorporated insecticide and nematocide carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) and combining traditional and molecular methods. In the first study, a closely timed sequence of treatments with analytical carbofuran was used to induce accelerated microbial degradation in four soils that had no previous exposure to carbofuran or other carbamate pesticides. Actual degradation rates of ^{14}C-carbonyl carbofuran (inactivation) and ^{14}C-ring carbofuran (mineralization) to $^{14}CO_2$ were measured over time to determine developmental patterns of accelerated microbial degradation. For all soils, accelerated microbial degradation occurred after one or two treatments. In the second study, treatments with analytical carbofuran were discontinued and the recovery patterns were determined. Recovery patterns of carbofuran inactivation were observed in three soils and recovery patterns of carbofuran mineralization were observed in two soils. To address present and future ecotoxicological

DEDICATION

Für Johannes

Mit Dir ist das Leben einfach schöner, ich liebe Dich.

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CHAPTER I

INTRODUCTION

Microbial degradation of pesticides in soil has generally been viewed as advantageous for the breakdown of potentially toxic residues (Jukes and Suett 1992). The persistence of chlorinated pesticides in the environment led to pressure from environmental organizations and the general public to develop degradable pesticides and to adopt agricultural practices which use lower dose rates (Jukes and Suett 1992). The replacement of recalcitrant pesticides with degradable, soil-applied pesticides in almost all markets set the stage for the development of accelerated microbial degradation (Racke 1990a). Therefore, the problem with newer pesticides is no longer the persistence of toxic residues in the soil, but rather loss of efficacy due to accelerated microbial degradation. Soils that exhibit this trait are often referred to as problem soils (Kaufman et al. 1985).

It has long been accepted that the degradative capacity of the soil microbial community is the most significant factor in determining pesticide efficacy of pesticides, particularly soil-applied pesticides. Accelerated microbial degradation develops when a pesticide is rapidly degraded by a population of microorganisms that has adapted due to previous exposure to it or a chemically similar pesticide (Racke and Coats 1988). In many cases, however, the development of accelerated microbial degradation has occurred after a single application of the recommended dose (Suett et al. 1996). In these cases, the pesticides are often

beneficial to soil microorganisms because they serve as a microbial carbon, energy or nutrient source (Racke and Coats 1988). Therefore, applying more than the recommended dose to remedy pest problems may actually exacerbate the problem. Accelerated microbial degradation can also persist for many years without further applications and can even affect the efficacy of other chemically-related pesticides that may be used in a pest management program (Jukes and Suett 1992). As used here, the term accelerated degradation refers to the microbial metabolism at rates that are 1) higher than those determined in previously untreated soils during development and field testing of the chemicals for registration and 2) high enough to seriously affect the efficacy of the compound when applied to soil (Anderson et al. 1998).

Accelerated microbial degradation of pesticides was first described in 1951 for the herbicide 2,4-D (Audus 1951). Before this time, failures of soil-applied insecticides to control target pests was usually attributed to improper application, unusual environmental conditions or the development of pest resistance (Racke 1990a). The problem of accelerated microbial degradation became a focus of researchers and extension workers in the corn belt of the United States in the mid-1970's (Suett 1990). At this time, loss of efficacy in the field performance of the herbicide EPTC and carbofuran were correlated with the rapid dissipation of their residues in previously-treated soils (Felsot 1989 and Tollefson 1986). Since then, hundreds of reports of accelerated microbial degradation of more than 50 different soil-applied pesticides have been published (Anderson et al. 1998).

Pesticides that have exhibited accelerated microbial degradation include: insecticides (e.g. Niemczyk and Chapman 1987, Felsot et al. 1981, Morel-Chevillet et al. 1996, Sethunathan and Pathak 1972), fungicides (e.g. Walker et al. 1986, Thom et al. 1997), herbicides (e.g. Andus 1951, Rahmann and James 1983, Skipper 1990, Hole and Powles 1997, Torstensson et al. 1975) and nematicides (e.g. Ou 1998, Smelt et al. 1989, Read 1986, Anderson 1989).

Accelerated microbial degradation of pesticides has had a significant impact on pesticide users, the research and extension community, and the agrochemical industry. Growers who rely on pesticides to meet their yield goals and ensure their livelihood are particularly affected (Racke 1990b). Accelerated microbial degradation has been the cause for numerous economically significant pest control failures in diverse crops such as corn, rice, sugar beets, onions, avocados and potatoes (Racke 1990b). These pest control failures also occur worldwide (Racke 1990b). Furthermore, the development of accelerated microbial degradation has led to a loss of confidence among growers in the ability of the agrochemicals industry to supply reliable and efficacious products (Racke 1990b).

Accelerated microbial degradation has had an adverse financial impact on the agrochemicals industry due to the loss of products or sales due to real or perceived pest control failures (Racke 1990b). Several products have subsequently been removed from the market or discontinued in late stages of

development, including fensulfothion (Chemagro), bufencarb (Chevron), bendiocarb (Fisons), cloethocarb (BASF), trimethacarb (Shell) and isofenphos (Mobay) (Felsot 1989, Racke 1990a). In Iowa, carbofuran (FMC) had 20% of the marketshare in 1977 and this declined to less than 8% by 1985 (Jennings and Stockdale 1978, Wintersteeen and Hartzler 1987).

The duration of the biological efficacy of a pesticide treatment is determined initially by the persistence of the biologically-active residue components followed by the extent to which these residues remain available to the target pest (Suett el al. 1996). Once accelerated microbial degradation has developed, the most effective management strategy is rotation of pesticides, and especially of pesticides belonging to different classes to prevent the development of cross-degradation (Suett 1990). Other strategies include using biocides, extenders and soil fumigants (Suett 1990). However, these methods may be expensive and not reliable (Suett 1990). Therefore, it is much more practical to suppress the development of accelerated degradation than to try to remedy the problem once it has developed.

There is a great need to be able to predict the development of accelerated microbial degradation of candidate pesticides early in the developmental process (Racke 1990b). In screening for potential new products, screening for the susceptibility of accelerated microbial degradation is a necessary step that no company can afford to ignore (Racke 1990b). Therefore, the main focus of this

study was to gain a greater understanding of the development of accelerated microbial degradation and its persistence in soils and to potentially use this information in the development of new pesticides. In addition, the applicability of novel methods molecular biology to serve as predictive tools of accelerated microbial degradation was examined. To address present and future ecotoxicological concerns, molecular methods were also used to assess possible effects of pesticide use on the soil microbial community.

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CHAPTER II

DEVELOPMENT OF ACCELERATED MICROBIAL DEGRADATION IN FOUR SOILS RETREATED WITH CARBOFURAN

Abstract

The development of carbofuran inactivation and mineralization rates was determined in four soils from Germany, Italy and New Zealand. The soil from Germany had corresponding field plots, and comparisons of carbofuran inactivation and mineralization were compared for this soil under field and laboratory conditions. All soils were treated with technical carbofuran every two months for six months. To measure the effects of these treatments, degradation rates of ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran to $^{14}CO_2$ in moist soil were determined over a 4 week period just prior to these treatments. Studies with ^{14}C-carbonyl carbofuran allowed determination of carbofuran inactivation, and studies with ^{14}C-ring carbofuran allowed determination of mineralization rates of carbofuran. All soils showed accelerated microbial degradation after one or two treatments with technical carbofuran. Therefore, the rapid development of microbial degradation in these soils merits consideration in developing effective pest management strategies. Furthermore, laboratory studies used in monitoring appeared conservative in estimating the development of accelerated microbial degradation.

Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum, systemic insecticide and nematocide used to control a range of pests including corn rootworm (*Diabrotica* spp., Felsot et al. 1985), cabbage root fly (*Phorbia* spp. Karpouzas et al. 2001) and carrot fly (*Psila* spp. Suett 1986) in a variety of agricultural crops such as rice, corn and beets (Morel-Chevillet et al. 1996). Accelerated degradation of carbofuran is well documented (e.g. Read 1986, Turco and Konopka 1990) and is an agricultural problem that arises when pesticides are repeatedly applied to the same soil resulting in reduced efficacy or failure of the compound (Anderson and Lafuerza 1992). As used here, the term accelerated degradation refers to the microbial metabolism at rates that are 1) higher than those determined in previously untreated soils during development and field testing of the chemicals for registration and 2) high enough to seriously affect or totally eliminate the efficacy of the compound when applied to soil (Anderson et al. 1998).

Several studies describe persistence of carbofuran in soils that do and do not have a treatment history (e.g. Turco and Konopka 1990, Talebi and Walker 1993). The majority of these focus on degradation and adsorption rates after a single treatment or multiple treatments of carbofuran have been discontinued. However, systematic studies that test the step-by-step increase in degradation potentials and rates of soils over time with retreatment are rare. This information is important to be able to predict dissipation rates after subsequent treatments to

avoid crop losses and to allow technical advisors in industry or extension services to accurately advise farmers. Furthermore, this information is of practical importance in establishing the potential degradative characteristics of individual soils prior to pesticide selection (Suett et al. 1996).

In this study, a closely timed sequence of treatments with carbofuran was used to induce accelerated microbial degradation to follow changes in soil degradation capacities over time. The objectives of this study were to: (1) evaluate changes of carbofuran inactivation and mineralization rates in four soils over time and (2) compare differences in carbofuran inactivation and mineralization rates between soil stored under laboratory conditions and a corresponding field plot.

Materials and Methods

Soil origin

Three soils from different geographic locations were used in this study. These soils were collected in Germany, Italy and New Zealand and none had previous treatment history of carbofuran or carbamate pesticides. Soil characterization data are presented in Table 2.1. Sites of collection are as follows:

Germany

Bayer Research Center, Monheim, Germany. Latitude: 51°4' N, longitude 6°55' E. Grass was planted in 1987, summer wheat in 1988, winter rye in 1988/89, winter barley in 1989/1990, winter wheat in 1990/1991, summer wheat in 1993,

winter rye in 1993/94, winter barley in 1995/95, winter wheat in 1995/96 and grass in summer 1996. The plot has been under grass and has not been treated with fertilizers since 1996. On March 7, 2000 the plot was plowed and then freshly planted with grass.

Italy

Greenhouse soil "Field B" from southern Italy: Apulien (region), Foggia (Province), Zapponeta (town), Torre Rivoli (location), Michele Piazzolla (farm owner). Latitude: 41°28' N, longitude 15°55' E. Distance from sea level = 0.5 m, distance from sea = 600 m.

New Zealand

The town closest to the field is Pukekohe, New Zealand. Latitude: 37°12' S, longitude: 174° 53' E.

Handling of laboratory soils

The soils from Italy and New Zealand were shipped to the Bayer Research Center in Monheim, Germany. Each sample was a mixed sample from at least 10 representative sites on each field or plot, as was the soil collected in Germany. Moisture conditions were not altered prior to shipping via air mail. Upon arrival in the laboratory, each soil was passed through a sterile, 2 mm sieve. Soils were stored in 10 l containers in the dark at 20°C.

Treatment of soil samples with technical carbofuran

Each soil was divided into two sections: untreated and treated. The treated soil was treated with technical carbofuran every 2 months. The treatment rate was agriculturally unacceptable, but pilot studies showed no toxic effects to microorganisms. Month 0 was May, 2001. Portions of each soil were removed and autoclaved twice, with 2 days between autoclavings. These were then placed in a drying oven. This dried soil was used to formulate technical carbofuran. The amount used for treatment of moist soil samples was 10 g dried soil / kg (dry wt.) of moist soil. This ratio remained constant. Technical carbofuran was dissolved in acetone and applied to the treated section. The untreated soil received the same amount of dried soil with acetone only. After the acetone had evaporated, the resulting carbofuran-soil formulation was used to treat the respective moist samples of soil. For this purpose, enough carbofuran-dried soil formulation was mixed with moist soil to give a final concentration of 4 mg carbofuran a.i. / kg dry wt of moist soil. This treatment rate (4 mg a.i./kg soil) was used throughout the experiment. Water was added to each soil so that the water content of the dried soil formulation was the same as the storage conditions.

Handling of field plots

Two plots at the Bayer Research Center, Monheim, Germany were utilized, each having an area of 1 m^2. The plots were in a row and had a buffer zone (grass) of 1.5 m. The control plot (untreated) was located at the highest elevation to avoid being affected by runoff from the treated plot.

The plots were routinely hoed to remove vegetation. The hoe was cleaned and sterilized with 70% ethanol after use in each plot. The untreated plot was hoed first, followed by the treated plot. To keep grass roots from entering the plots, the outer borders were delineated by inserting 4 running meters of corrugated plastic sheeting (2 mm thick, 19.5 cm high) to a depth of ca. 10 cm around the outer edges of the 1 x 1 m^2 plant-free plots. To keep small animals and birds off of the soil, each plot was covered by a 1 m^2 metal grid with spaces of ca. 2 cm^2.

For treatments with technical carbofuran every two months, ca. 3 kg of soil from the upper 10-20 cm of each plot was removed and placed in clean, labeled bags. The soil samples were brought to the lab and passed through alcohol sterilized and flamed 2mm sieves.

Two kg dry weight soil from the treated plot was treated with technical carbofuran. To formulate technical carbofuran, 50 g of dried soil was weighed into drying dishes. This dried soil was prepared the same as the laboratory soils described above. A solution containing 10 mg a.i. in 20 ml acetone was

prepared. This was transferred to the dried soil and allowed to evaporate. After the acetone had evaporated, the dried soil was thoroughly mixed with a spoon spatula, and a portion of 20 g was weighed into separate dishes (10 g air-dried soil / 1 kg dry wt. soil, 4 mg a.i./ kg dry wt soil). For treatment of the control plot, 10 ml acetone was applied to a 20 g portion of dried soil.

The 20 g portions of carbofuran formulated on dried soil were used to treat the appropriate kg dry wt. soil batch of moist, sieved soil. To do this, the formulation was added to the 2 kg portion of moist soil and was mixed by tumbling diagonally on a rotary mixer for 15 minutes. The control soil was handled in the same manner with the 20 g dried soil without technical carbofuran.

The treated soils were taken back to the field and evenly distributed across the surface appropriate plot. The treated soils were mixed into the upper 5 cm of the plot by hoeing.

Throughout the course of this study, the vegetation on the plots was regularly removed to avoid absorption of carbofuran by the plants.

Pesticide

Pure analytical (chemical purity > 99%) and radiochemical (radiochemical purity > 99%) carbofuran (2,2-dimethyl-2,3-dihydrobenzo-furanyl-7-methylcarbamate) were supplied by Bayer CropScience (Monheim, Germany). ^{14}C-carbonyl carbofuran from stock solution (specific activity = 4.03 MBq/mg) was mixed with 0.12 g technical material to give a treating solution with a final specific activity of 0.833 KBq/mg. ^{14}C-ring carbofuran from stock solution (specific activity = 2.54 MBq/mg) was mixed with 0.12 g technical material to give a treating solution with a final specific activity of 8.33 KBq/mg. Both ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran treating solutions were made with acetone and had concentrations of 600 µg active ingredient per 50 µl. Tests with ^{14}C-carbonyl carbofuran allowed evaluation of the intensity of degradation and inactivation of the chemical by release of the carbonyl moiety (Talebi and Walker 1993). Tests with ^{14}C-ring carbofuran allowed evaluation of the rate of mineralization of the carbofuran metabolite, carbofuran phenol (Talebi and Walker 1993).

Radioactive tests

Degradation rates of ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran to $^{14}CO_2$ in moist soil were determined over a four week period just prior to treatments with technical carbofuran to determine their effects. For these tests, two portions each of 30 g dry weight soil were removed from each soil group (untreated and treated) and treated with either ^{14}C-carbonyl carbofuran or ^{14}C-ring carbofuran formulated on quartz sand. The final amount of chemical added to each 30 g dry

weight portion was 900 µg. After thoroughly mixing, 3 portions of 10 g dry wt. soil were removed and placed in 20 ml scintillation bottles. The water content was then to ca. 40 % water capacity for all samples. Each scintillation bottle with soil was placed in a separate 250 ml brown glass jar along with an additional 20 ml scintillation bottle containing 5 ml 1 N NaOH. The brown jars were sealed tightly with lids and incubated at 20 $\pm$ 2 °C in the dark. NaOH was replaced weekly, thereby also aerating the bottles. $^{14}CO_2$ evolution was determined by mixing the removed NaOH with 7 ml scintillation cocktail (Quickszint 401, Zinsser Analytic, Berkshire, UK). The amount of radioactivity determined by liquid scintillation counting (LSC) for 10 minutes in a LKB-Wallac 1219 Spectral liquid scintillation analyzer (Perkin Elmer Wallac GmbH, Freiburg, Germany).

Sterile radioactive tests and release of bound CO_2

Degradation rates of ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran to $^{14}CO_2$ in sterile moist soils were measured over a four week period to determine how much of carbofuran degradation is due to abiotic processes and how much is due to volatilization. These tests were conducted under the sterile hood, and all supplies mentioned were sterilized by autoclaving. First, two portions each of 30 g dry weight soil were removed from each soil group (untreated and treated) and weighed into separate 1 l glass beakers. The beakers were covered with aluminium foil, labeled accordingly, and the weights of the beakers were recorded. After the soils were autoclaved twice with two days in-between, they were placed in a drying oven at 80°C for approximately three hours. Afterwards,

the weights of the beakers were recorded, and the samples were adjusted to 40% water capacity. ^{14}C-carbonyl carbofuran or ^{14}C-ring carbofuran were formulated on quartz sand, yielding final concentrations of 900 µg for each 30 g dry weight portion of soil. After thoroughly mixing, 3 portions of 10 g dry wt. soil were removed and placed in 20 ml glass scintillation bottles, and a cotton ball was placed on each sample to prevent contamination. The samples were placed in separate 250 ml glass jars. Five milliliters of 1 M NaOH were transferred to modified test tubes, i.e. holes on the side to allow gas exchange and with stoppers to hang on the rim of the 250 ml bottle. These modified test tubes were then placed on each 250 ml glass jar and tightly sealed with lids. All samples were incubated at 20°C in the dark. Every week for four weeks, NaOH was removed using sterile pipette tips and transferred to a new scintillation bottle. $^{14}CO_2$ evolution was determined by mixing the removed NaOH with 7 ml scintillation cocktail (Zinsser Analytic, Quickszint 401). The amount of radioactivity determined by liquid scintillation counting (LSC) for 10 minutes in a LKB-Wallac 1219 Spectral liquid scintillation analyzer (Perkin Elmer Wallac GmbH). Five milliliters of fresh 1 N NaOH were transferred into each modified test tube.

After four weeks, sterility of the samples was checked by placing several soil particles from each sample onto separate malt agar plates (ca. 50 g/ l). After the soil samples were plated out, 6 ml of 1% HCl was added to each soil sample to extract bound $^{14}CO_2$. Immediately afterwards, the modified test tubes with 5 ml

fresh 1 N NaOH were placed on the 250 ml bottles, which were sealed tightly with lids. After one week, the NaOH was transferred to separate scintillation bottles, 7 ml scintillation cocktail (Zinsser Analytic, Quickszint 401) was added to each sample, and the amount of radioactivity determined by liquid scintillation counting (LSC) for 10 minutes in a LKB-Wallac 1219 Spectral liquid scintillation analyzer (Perkin Elmer Wallac GmbH).

Soil microbial biomass: Substrate Induced Respiration (SIR)

Triplicate soil subsamples (25 g) for each soil and soil treatment group were placed in 1 ml glass beakers. A mixture of 0.075 g glucose and ca 0.5 g quartz sand was added to each sample and thoroughly mixed by hand. Each glucose-amended soil sample was transferred to a plastic cylinder, with sponge plugs on both ends. The cylinders were connected to a Ultragas 3B CO_2-analyzer (Wösthoff, Bochum, Germany), incubated at 20°C, and mg CO_2 released per hour was quantified for up to 10 hours. The substrate induced respiration rate was expressed as mg microbial C / kg dry wt. soil (Anderson and Domsch 1978, Anderson 1982). Preliminary experiments indicated that the addition of 0.003 g glucose g^{-1} soil resulted in maximal rates of SIR during the incubation period.

Data analysis

Statistical analysis of the effects of soil and treatments over time was conducted using an ANOVA and the proc mixed function in SAS as described by Littell et al. (1996). The repeated/group option of proc mixed was used whenever the assumption of homogeneity of variances was violated. Differences between a given week across months and between weeks during a monthly interval were determined using slicing and pairwise comparisons. The same level of significance ($p < 0.05$) was used to assess statistical significance in all the analyses.

Results and Discussion

Comparison of soils stored under laboratory conditions: inactivation

The soil from New Zealand and the soil from Germany stored under laboratory conditions showed accelerated rates of carbofuran inactivation after a single treatment with technical carbofuran (month 2) (Table 2.2). The soil from Italy had accelerated rates of carbofuran inactivation after two treatments with technical carbofuran (month 4) (Table 2.2). These results are in accordance with other studies that found similar rates in the loss of carbofuran efficacy in agricultural soils (Camper 1987, Talebi and Walker 1993). Once accelerated degradation rates were observed, the levels remained relatively constant for each soil (Table 2.2). This suggests microbial adaptation to carbofuran was the determining factor for accelerated degradation rates as the soil microbial community had become adapted to the pesticide. Moreover, once accelerated degradation

occurred, most of the dissipation of carbofuran occurred during the first week of monitoring for the respective monthly interval (Table 2.2).

The lower levels of active microbial biomass in the soil from Italy compared to the soils from New Zealand and the soil from Germany stored under laboratory conditions may explain why explain why accelerated rates of inactivation developed more slowly in this soil (Figures 2.1-2.3). For all soils, the level of active microbial biomass remained constant or slightly decreased over time so the development of accelerated microbial degradation in these soils was probably not due to an increase in the total microbial community (Figures 2.1-2.4). These results are in accordance with other studies where accelerated degradation was not correlated with an increase in microbial biomass (Turco and Konopka 1990).

Comparison of soils stored under laboratory conditions: mineralization

All soils exhibited accelerated rates of carbofuran mineralization except the soil from Italy for which almost no mineralization occurred (Table 2.3). The lack of carbofuran mineralization in the soil from Italy may also be due to its low levels of active microbial biomass (Figure 2.1). Furthermore, mineralization of carbofuran has also been found to be a cometabolic process (Trabue et al. 2001).

The appearance of accelerated rates of carbofuran mineralization in the soil from New Zealand and the soil from Germany stored under laboratory conditions

coincided with the appearance of accelerated rates of carbofuran inactivation (Tables 2.2 and 2.3). As with the development of carbofuran inactivation, almost all of the mineralization of carbofuran occurred during the first week of monitoring for the respective monthly interval (Table 2.3). In all soils, the rates of carbofuran mineralization were significantly lower than the rates of carbofuran inactivation. These results are supported by other studies that found carbofuran inactivation rates of 68% to 73% but that mineralization rates in the same soils was minimal (Turco and Konopka 1990 and Talebi and Walker 1993). This would be expected since the first step in carbofuran metabolism in soil is via hydrolysis to form carbofuran phenol and methylamine (Ou et al. 1982, Trabue et al. 2001). Carbofuran phenol is further metabolized by cleavage of the aromatic ring and ultimately leads to the formation of CO_2 and H_2O (Ou et al. 1982, Trabue et al. 2001).

Abiotic degradation

A significant amount of carbofuran inactivation due to abiotic processes and volatilization was only observed in the soil from Italy (Table 2.4). After four weeks, the amount of carbofuran inactivated in this soil was 33.45 ± 2.66% (Table 2.4). Inactivation rates in the soils from New Zealand and Germany were approximately 1% or less (Table 2.4). These results support published evidence carbofuran can be degraded abiotically, but chemical hydrolysis contributes to dissipation only in alkaline soils (Getzin 1973, Charnay and Fournier 1994). Rates of carbofuran mineralization due to abiotic processes and volatilization

were less than 1% after four weeks of monitoring for all soils (data not shown) indicating the accelerated mineralization rates observed in the soil from New Zealand and the soil from Germany stored under laboratory conditions were almost entirely due to microbial processes.

Comparison of field and laboratory soils from Germany

The field soil from Germany developed accelerated rates of carbofuran inactivation after two treatments with technical carbofuran, which corresponds to month four of the study (Table 2.2). Compared to compared to the soil from Germany stored under laboratory conditions, this development occurred one treatment later (Table 2.2). The development of accelerated mineralization rates in the field soil from Germany also occurred after an additional treatment compared to the soil from Germany stored under laboratory conditions (Table 2.3).

The cumulative degradation rates of carbofuran inactivation and mineralization, respectively, were approximately the same for these soils after two treatments with technical carbofuran (Tables 2.2 and 2.3). However, the field soil showed a lag time during the four weeks of monitoring during this monthly interval, whereas the soil stored under laboratory conditions showed almost all of the degradation occurring during week one (Tables 2.2 and 2.3). Rates of carbofuran inactivation and mineralization were more similar for these soils after three treatments with

technical carbofuran, which corresponds to month six of the study (Tables 2.2 and 2.3).

As with the soil stored under laboratory conditions, the field soil also did not show an increase in the active microbial biomass over time (Figure 2.4). However, due to seasonal fluctuations, the soil microbial population in the field soil may be more subject to changes in its composition thereby affecting its response to pesticide treatments (Suett et al. 1996 and Ou et al. 1982). The field soil may also have showed slower development of carbofuran inactivation and mineralization since its exposure to carbofuran may have been decreased by environmental effects such as leaching.

Conclusions

In conclusion, microbial activity was the main cause for loss of carbofuran efficacy in these soils. All soils exhibited accelerated rates of carbofuran inactivation and mineralization after one or two treatments with technical carbofuran. Therefore, the rapid development of microbial degradation in these soils merits consideration in developing effective pest management strategies. Furthermore, laboratory studies used in monitoring may be conservative in estimating the development of accelerated microbial degradation.

Origin	Description	% Water Content	$pH_{(KCl)}$	% Organic C	% Total N
Germany (lab)	Silty Sand	8.3	5.65	0.6	0.08
Germany (field)	Silty Sand	variable	5.65	0.6	0.08
Italy	Sand	7.4	8.44	2.5	0.06
Zew Zealand	Clay Loam	34.1	4.95	4.0	0.35

Table 2.1: Background information of soils used in this study.

Cumulative % Applied ^{14}C-CO Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks						
Soil Origin	**Month**	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Germany (Laboratory)	0	None	5.4 ± 0.1	11.3 ± 0.2	16.8 ± 0.3	21.9 ± 0.5
		Every 2 Months	4.8 ± 1.1	10.1 ± 2.2	14.7 ± 3.3	19.5 ± 3.4
	2	None	3.0 ± 0.8	6.3 ± 0.8	9.8 ± 0.7	13.3 ± 0.5
		Every 2 Months	77.6 ± 10.7	78.4 ± 10.6	78.7 ± 10.6	78.8 ± 10.7
	4	None	2.1 ± 0.0	4.9 ± 0.2	7.4 ± 0.4	9.3 ± 0.4
		Every 2 Months	80.0 ± 3.6	80.7 ± 3.6	81.0 ± 3.6	84.8 ± 3.3
	6	None	2.4 ± 0.1	4.5 ± 0.0	6.6 ± 0.0	8.6 ± 0.1
		Every 2 Months	81.4 ± 6.1	82.0 ± 6.1	82.3 ± 6.1	82.4 ± 6.1
Italy	0	None	19.1 ± 4.3	39.6 ± 7.1	56.1 ± 7.6	67.5 ± 7.7
		Every 2 Months	21.5 ± 1.9	43.8 ± 4.7	60.2 ± 6.2	71.5 ± 5.9
	2	None	22.4 ± 3.2	43.2 ± 7.0	51.9 ± 5.8	62.4 ± 6.3
		Every 2 Months	24.1 ± 0.4	45.7 ± 1.8	54.8 ± 1.3	64.2 ± 2.6
	4	None	26.0 ± 1.1	47.8 ± 5.3	50.0 ± 5.2	59.9 ± 5.5
		Every 2 Months	62.5 ± 8.3	75.6 ± 3.7	76.4 ± 3.7	78.0 ± 3.4
	6	None	22.9 ± 2.6	41.8 ± 3.2	56.1 ± 3.2	67.0 ± 3.6
		Every 2 Months	71.2 ± 5.6	79.2 ± 5.7	81.3 ± 5.8	82.5 ± 5.9
New Zealand	0	None	1.6 ± 0.2	3.8 ± 0.2	6.5 ± 0.9	9.4 ± 1.7
		Every 2 Months	1.4 ± 0.2	3.6 ± 0.4	6.0 ± 0.7	8.5 ± 1.0
	2	None	1.9 ± 0.2	6.1 ± 0.2	8.6 ± 0.2	11.6 ± 0.3
		Every 2 Months	87.8 ± 6.9	94.6 ± 6.1	95.7 ± 6.1	96.3 ± 6.0
	4	None	1.4 ± 0.1	3.6 ± 0.1	5.4 ± 0.1	7.1 ± 0.1
		Every 2 Months	71.4 ± 4.1	71.5 ± 4.2	72.8 ± 4.4	73.3 ± 4.5
	6	None	1.2 ± 0.0	2.6 ± 0.0	4.2 ± 0.0	6.0 ± 0.1
		Every 2 Months	85.6 ± 2.6	88.9 ± 2.9	90.2 ± 3.1	91.0 ± 3.5
Germany (Field Plot)	0	None	4.5 ± 0.4	10.0 ± 0.8	15.0 ± 1.3	19.5 ± 1.5
		Recovery	4.5 ± 0.1	9.8 ± 0.1	14.6 ± 0.2	18.8 ± 0.3
	2	None	5.8 ± 0.2	9.2 ± 0.1	12.9 ± 0.1	16.2 ± 0.2
		Recovery	5.5 ± 0.4	9.5 ± 0.5	13.8 ± 0.5	17.5 ± 0.5
	4	None	1.6 ± 0.2	17.0 ± 0.5	19.9 ± 0.7	23.4 ± 1.6
		Recovery	13.8 ± 0.4	42.4 ± 24.5	82.0 ± 6.5	84.9 ± 4.7
	6	None	3.4 ± 0.1	7.6 ± 0.2	12.1 ± 0.4	15.7 ± 0.5
		Recovery	81.7 ± 7.9	97.1 ± 5.0	97.4 ± 4.5	97.5 ± 4.3

Table 2.2: Carbofuran inactivation rates measured as cumulative percent of applied ^{14}C-carbonyl carbofuran degraded to $^{14}CO_2$ over four weeks. Values are the averages of three replicate samples and standard deviations are also given.

Cumulative % Applied ^{14}C-ring Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks						
Soil Origin	**Month**	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Germany (Laboratory)	0	None	0.7 ± 0.1	1.4 ± 0.2	2.3 ± 0.3	3.3 ± 0.3
		Every 2 Months	0.6 ± 0.1	1.4 ± 0.3	2.4 ± 0.3	3.4 ± 0.3
	2	None	0.5 ± 0.1	0.9 ± 0.2	1.5 ± 0.2	2.1 ± 0.3
		Every 2 Months	44.8 ± 6.9	48.8 ± 6.1	50.1 ± 5.9	51.0 ± 5.8
	4	None	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	1.6 ± 0.0
		Every 2 Months	39.6 ± 2.9	44.0 ± 3.2	45.9 ± 3.4	50.0 ± 2.9
	6	None	0.6 ± 0.0	1.0 ± 0.0	1.3 ± 0.0	1.6 ± 0.0
		Every 2 Months	48.7 ± 2.0	53.6 ± 2.2	56.0 ± 2.3	57.3 ± 2.2
Italy	0	None	0.4 ± 0.2	0.9 ± 0.5	1.5 ± 0.8	2.2 ± 1.2
		Every 2 Months	0.4 ± 0.1	1.0 ± 0.2	1.7 ± 0.3	2.5 ± 0.3
	2	None	0.7 ± 0.0	1.5 ± 0.0	1.7 ± 0.0	2.7 ± 0.0
		Every 2 Months	0.6 ± 0.1	1.4 ± 0.2	2.5 ± 0.4	3.5 ± 0.4
	4	None	0.8 ± 0.0	1.7 ± 0.1	2.6 ± 0.1	3.5 ± 0.2
		Every 2 Months	1.3 ± 0.0	2.1 ± 0.0	2.9 ± 0.0	3.6 ± 0.4
	6	None	0.7 ± 0.0	1.1 ± 0.2	1.8 ± 0.2	2.6 ± 0.2
		Every 2 Months	0.7 ± 0.4	1.3 ± 0.8	1.8 ± 1.1	2.4 ± 1.4
New Zealand	0	None	0.3 ± 0.1	0.6 ± 0.0	1.1 ± 0.2	1.7 ± 0.4
		Every 2 Months	0.3 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.8 ± 0.1
	2	None	0.4 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	1.9 ± 0.1
		Every 2 Months	40.4 ± 5.3	40.7 ± 5.3	44.9 ± 5.3	47.9 ± 5.3
	4	None	0.6 ± 0.0	0.9 ± 0.0	1.3 ± 0.0	1.8 ± 0.1
		Every 2 Months	39.0 ± 0.7	39.7 ± 0.7	41.9 ± 0.7	43.3 ± 0.7
	6	None	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	1.5 ± 0.2
		Every 2 Months	39.5 ± 3.2	43.1 ± 3.9	45.1 ± 4.3	46.6 ± 4.5
Germany (Field Plot)	0	None	0.8 ± 0.1	1.7 ± 0.1	2.7 ± 0.2	3.8 ± 0.2
		Recovery	0.7 ± 0.0	1.6 ± 0.0	2.6 ± 0.1	3.6 ± 0.1
	2	None	0.0 ± 0.0	0.7 ± 0.0	1.5 ± 0.0	2.4 ± 0.0
		Recovery	0.0 ± 0.0	0.6 ± 0.0	1.5 ± 0.0	2.2 ± 0.0
	4	None	0.7 ± 0.0	1.4 ± 0.0	2.1 ± 0.0	2.9 ± 0.1
		Recovery	0.6 ± 0.0	2.1 ± 0.6	23.4 ± 6.6	40.3 ± 10.1
	6	None	0.8 ± 0.0	1.5 ± 0.0	2.4 ± 0.0	3.2 ± 0.1
		Recovery	31.3 ± 9.3	50.7 ± 3.5	54.4 ± 3.3	56.1 ± 3.5

Table 2.3: Carbofuran mineralization rates measured as cumulative percent of applied ^{14}C-ring carbofuran degraded to $^{14}CO_2$ over four weeks. Values are the averages of three replicate samples and standard deviations are also given.

Soil Origin	pH	Cumulative % of Applied ^{14}C-carbonyl Carbofuran Degraded to $^{14}CO_2$ in Sterile Soils				Degradation after HCl Addition	pH after HCl Addition
		Week 1	Week 2	Week 3	Week 4		
Germany	5.65	0.24 ± 0.01	0.51 ± 0.04	0.75 ± 0.03	1.00 ± 0.05	1.16 ± 0.07	2.17
Italy	8.44	9.16 ± 0.19	21.33 ± 1.37	28.53 ± 2.43	33.45 ± 2.66	48.48 ± 2.55	6.67
New Zealand	4.95	0.17 ± 0.01	0.26 ± 0.02	0.33 ±0.01	0.42 ± 0.02	0.49 ± 0.02	3.51

Table 2.4: Carbofuran inactivation rates measured in sterile soil as cumulative percent of applied ^{14}C-carbonyl carbofuran degraded to $^{14}CO_2$ over four weeks. Values are the averages of three replicate samples and standard deviations are also given.

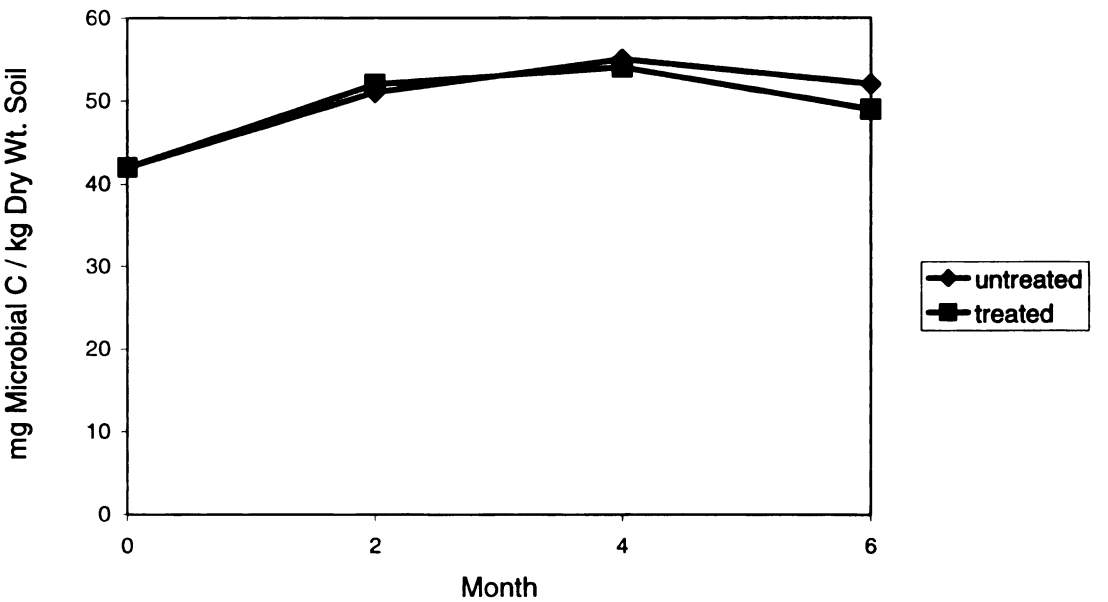

Figure 2.1: Active microbial biomass expressed as mg microbial C/ kg dry wt. soil for the soil from Italy.

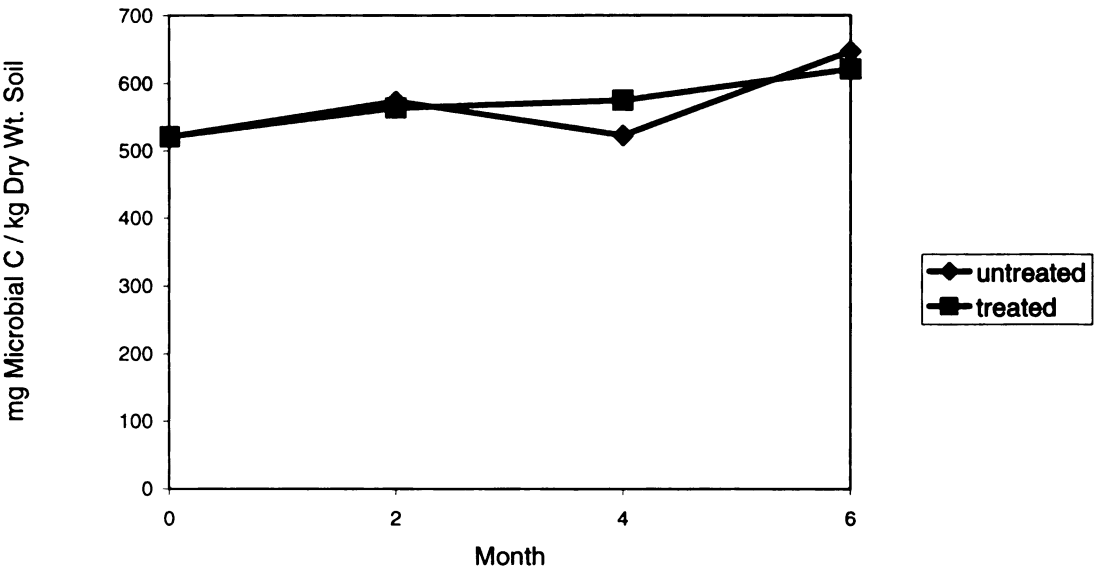

Figure 2.2: Active microbial biomass expressed as mg microbial C/ kg dry wt. soil for the soil from New Zealand.

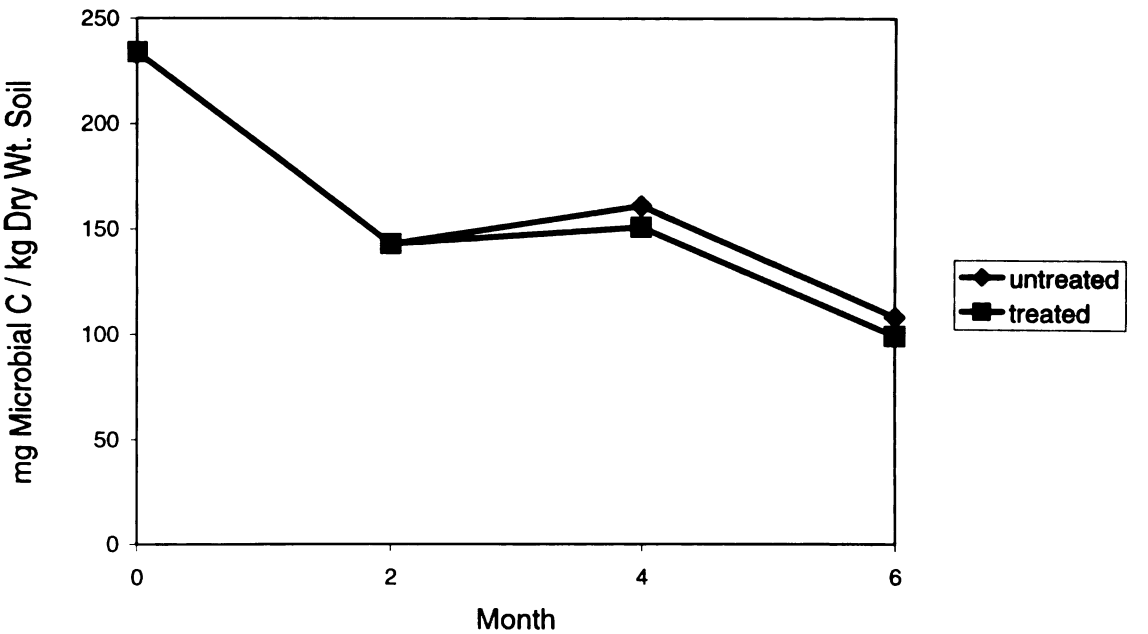

Figure 2.3: Active microbial biomass expressed as mg microbial C/ kg dry wt. soil for the soil from Germany stored under laboratory conditions.

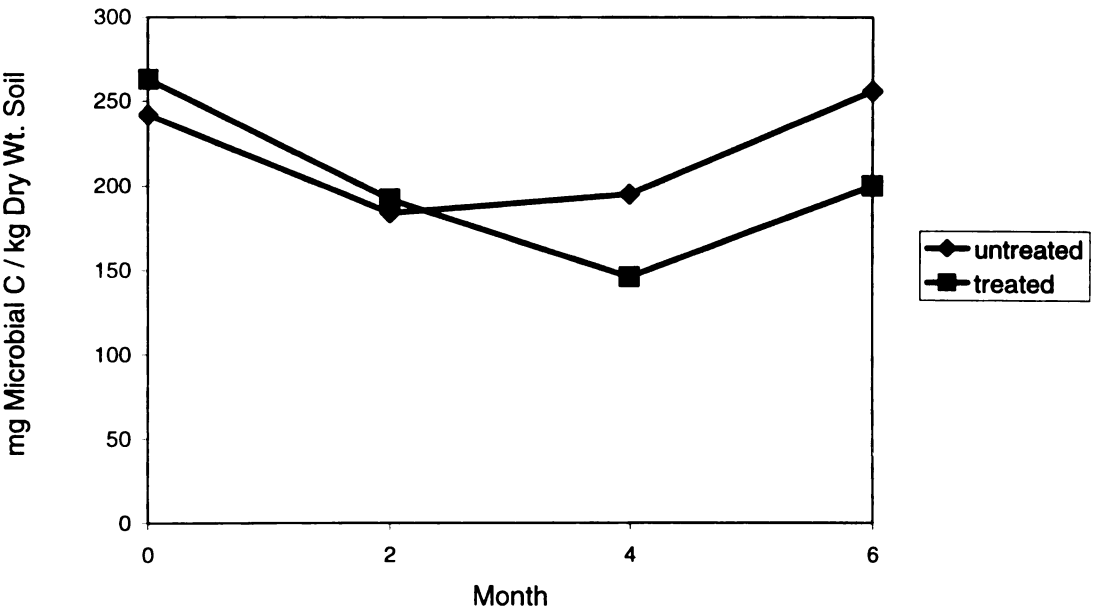

Figure 2.4: Active microbial biomass expressed as mg microbial C/ kg dry wt. soil for the field soil from Germany.

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CHAPTER III

DECELERATION OF CARBOFURAN DEGRADATION RATES IN FOUR SOILS THAT HAD SHOWN ACCELERATED MICROBIAL DEGRADATION

Abstract

The capacity of four soils to inactivate and mineralize carbofuran was determined using slurry conditions. These results were combined with the active microbial biomass measured by substrate induced respiration to obtain the amount of carbofuran degraded per mg microbial carbon. These soils had exhibited accelerated rates of carbofuran degradation and were from Germany, Italy and New Zealand. The soil from Germany had corresponding field plots, and comparisons of carbofuran inactivation and mineralization were compared for this soil under field and laboratory conditions. In addition, the deceleration of carbofuran inactivation and mineralization rates, also known as recovery, was determined for these soils. All soils were systematically monitored for 16 months after treatments with analytical carbofuran had been discontinued. Specifically, degradation rates of ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran to $^{14}CO_2$ in moist soil were determined over a four week period every two months. All soils, except the soil from Germany stored under laboratory conditions showed recovery patterns of carbofuran inactivation. Two soils, the soil from New Zealand and the field soil from Germany, showed signs of recovery from carbofuran mineralization. Therefore, the field soil and the soil stored under laboratory conditions showed significant differences in rates of carbofuran

inactivation and mineralization. No distinct correlation was seen in these soils between their actual carbofuran degradation rates and their capacity to degrade carbofuran.

Introduction

Accelerated microbial degradation occurs when microorganisms adapt to repeated pesticide applications to soil (Anderson et al. 1998). Felsot et al. (1981) conducted one of the first studies that demonstrated the development of accelerated microbial degradation of carbofuran in soils that had a history of carbofuran application. Since then, numerous studies have described accelerated microbial degradation of carbofuran in soils with and without carbofuran treatment history (e.g. Turco and Konopka 1990, Talebi and Walker 1993).

The persistence of accelerated microbial degradation in soil is a problem that precludes the efficient use of carbofuran in a pest management program. This persistence may last for different periods of time depending on soil type and other abiotic factors (Racke and Coats 1990). Accelerated microbial degradation of carbofuran was sustained in agricultural soils from the United Kingdom without additional applications for over five years (Suett et al. 1993). Accelerated microbial degradation also persisted in various agricultural soils that had a single application of carbofuran for up to four years (Eagle 1986). These studies

measured the degradation rates after a hiatus since the initial determination of accelerated degradation.

Patterns of deceleration rates, usually referred to as recovery rates, obtained from systematic, repeated studies over time are critical in determining the duration of accelerated microbial degradation so that effective pesticide management strategies can be developed accordingly. These patterns of recovery rates also have ramifications for determining ultimate mineralization of pesticides in the soil environment. To our knowledge, only one study exists that systematically and repeatedly measured the deceleration capacity of degradation rates in soils that previously showed accelerated microbial degradation rates of a pesticide. This study was conducted with agricultural field soils that had showed accelerated microbial degradation of the organophosphate nematocide fenamiphos (Anderson and Lafuerza 1992).

Recovery rates differ between pesticides and therefore must be determined separately for each pesticide (Anderson and Lafuerza 1992). This information is important in determining when soils return to degradation rates found in previously untreated soils. If the pesticide is applied too soon, it will lead to rapid re-development of new degrading populations that will result in renewed decrease in efficacy (Anderson and Lafuerza 1992).

Therefore, the objectives of this study were to systematically and repeatedly: 1) determine the capacity of the active microbial biomass in four soils showing accelerated microbial degradation of carbofuran to inactivate and mineralize carbofuran, 2) determine the recovery patterns of carbofuran inactivation and mineralization in four soils shown to exhibit accelerated microbial degradation of carbofuran and 3) determine whether the soil from the field responded similarly to soils monitored under laboratory conditions to evaluate the applicability of laboratory studies in predicting field response.

Materials and Methods

Pesticide

Pure technical (chemical purity > 99%) and radiochemical (radiochemical purity > 99%) carbofuran (2,2-dimethyl-2,3-dihydrobenzo-furanyl-7-methylcarbamate) were supplied by Bayer CropScience (Monheim, Germany). Two isotopes of ^{14}C-carbofuran were used. Tests with ^{14}C-carbonyl carbofuran allow evaluation the inactivation of the chemical by release of the carbonyl moiety (Talebi and Walker 1993). Tests with ^{14}C-ring carbofuran allow evaluation of the rate of mineralization of the carbofuran (Talebi and Walker 1993). Specific activities of radiochemical stock and treating solutions are given in Chapter 2.

Soils

The four soils used in this study are described in Chapter 2. These soils are from New Zealand (clay loam, pH = 4.95), Italy (sand, pH = 8.44) and Germany (silty sand, pH = 5.65). The soil from Germany was divided into two groups; one portion was taken to be stored under laboratory conditions and the rest remained in the field. Since this soil was handled differently, the soil in the field and in the laboratory are considered to two separate soils. Each of the four soils was further subdivided into three different treatment groups:

A—never treated with technical carbofuran, negative control

B—treated every two months with analytical carbofuran for six months at which time treatments were discontinued and the current study began, recovery

C—treated every two months with analytical carbofuran for the duration of the study, positive control.

Soil microbial biomass

The substrate induced respiration method was used to determine the active microbial biomass in soil samples (Anderson and Domsch 1978, Anderson 1982). Specific conditions of this study are described in Chapter 2. Briefly, triplicate soil samples were treated with glucose and incubated at 20°C. The amount of CO_2 released per hour was quantified for up to 10 hours. The amount of active microbial biomass was expressed as mg microbial C / kg dry wt. soil.

Degradation capacity

The capacity of *in situ* soil microorganisms to degrade ^{14}C-carbonyl and ^{14}C-ring carbofuran was determined as described by Anderson and Lafuerza (1992). Briefly, subsamples of soil were mixed with 120 mg ^{14}C-carbofuran/ kg dry wt. soil and incubated in the dark under slurry conditions on a shaker (140 rpm) at 25°C for seven days. The slurry conditions allow maximum bioavailability of carbofuran to microorganisms. Hence, the carbofuran degradation capacity, i.e. the µg carbofuran degraded / mg microbial carbon after seven days, was determined as described by Anderson et al. (1998).

Actual degradation rates

To determine the actual rates of carbofuran inactivation and mineralization, i.e. the effects of treatments with analytical carbofuran, degradation rates of ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran to $^{14}CO_2$ in moist soil were determined over a 4 week period just prior to the treatments with analytical carbofuran. For this, moist soil was incubated at 20° C in the dark and the specific protocol is described in Chapter 2. Soils were monitored for 16 months after treatments with analytical carbofuran were discontinued in the B (recovery) groups. The moist soil incubation method is a more accurate indication of field degradation rates since it more closely resembles field conditions compared to the slurries described above. Therefore, this method will be used to obtain deceleration patterns of carbofuran inactivation and mineralization.

Data analyses

Statistical analysis of the effects of soil and treatments over time was conducted using an ANOVA and the proc mixed function in SAS as described by Littell et al. (1996). The repeated/group option of proc mixed was used whenever the assumption of homogeneity of variances was violated. Differences between a given week across months and between weeks during a monthly interval were determined using slicing and pairwise comparisons. The same level of significance ($p < 0.05$) was used to assess statistical significance in all the analyses.

Results and Discussion

Soil microbial biomass

The active microbial biomass between treatment groups within soils responded similarly over time (Figures 3.1-3.4). The soil from New Zealand had the highest level of active microbial biomass (400-700 mg microbial C/ kg dry wt. soil) and the soil from Italy had the lowest level of active microbial biomass (30-50 mg microbial C/ kg dry wt. soil) (Figures 3.1 and 3.4). The active microbial biomass in the soils from Germany and Italy remained constant over time and decreased slightly in the soil from New Zealand (Figures 3.1, 3.3 and 3.4). This indicates the observed recovery rates of carbofuran degradation in some soils resulted from other mechanisms than a considerable decrease in the total active microbial biomass (Tables 3.1-3.8). Conversely, the persistence of accelerated microbial

degradation in other soils lasts due to other mechanisms than a considerable increase in the total active microbial biomass (Tables 3.1-3.8).

Non-systematically repeated studies on the effect pesticide application on the microbial biomass reported mixed results. A study with the herbicides 2,4-D and atrazine did not show a correlation between microbial biomass and degradation rates (Entry et al. 1994, Entry and Emmingham 1996, Ghani et al. 1996). Propanil appeared not to have a significant effect on the bacterial community although HPLC showed it disappeared rapidly (Crecchio et al. 2001). However, evidence exists that pesticide application may positively correlate to changes in microbial biomass. For example, SIR measurements positively and significantly correlated with degradation rates of the fungicide metalaxyl and the herbicide propachlor in various agricultural soils (Jones and Ananyeva 2001). One study with carbofuran indicated accelerated degradation rates of carbofuran were associated with initial increase in microbial populations, but approximately three weeks after treatment the total soil microbial populations were equal or lower to populations in control soils without pesticide application (Edwards et al. 1992). The herbicide dinoterb caused a 22% decrease in microbial biomass as measured with SIR (Engelen et al. 1998).

Degradation capacity: carbofuran inactivation

The capacity of the active microbial biomass in these soils to inactivate carbofuran, i.e. convert ^{14}C-carbonyl carbofuran to $^{14}CO_2$ using slurry conditions, is shown in Figures 3.5-3.8. The capacity of the active microbial biomass in the soil from New Zealand and the two soils from Germany to inactivate carbofuran varies between groups B and C over time, indicating the B groups of these soils are less capable of inactivating carbofuran and start to show signs of recovery (Figures 3.5 and 3.6). The capacity of the B group of the field soil from Germany to inactivate carbofuran almost returned to levels of the A group, whereas the B group from the soil from New Zealand and the soil from Germany stored under laboratory conditions did not (Figures 3.5 and 3.6). Again, the A group refers to the group of soil that never received treatments with analytical carbofuran, the B group received treatments with analytical carbofuran every two months for six months prior to the current study and the C group continually received treatments with analytical carbofuran every two months. The active microbial population in the soil from Italy was able to inactivate more µg carbofuan per mg microbial C compared to the other soils (Figure 3.8). No distinction was evident between treatment groups in this soil (Figure 3.8).

Degradation capacity: carbofuran mineralization

The capacity of the active microbial biomass in these soils to mineralize carbofuran, i.e. convert ^{14}C-ring carbofuran to $^{14}CO_2$ using slurry conditions, is shown in Figures 3.9-3.12. The capacity of the active microbial biomass in the

soil from New Zealand and the field soil from Germany to mineralize carbofuran yield similar trends compared to their capacity to inactivate carbofuran (Figures 3.2 and 3.10). For all soils, the capacity to mineralize carbofuran was lower than their capacity to inactivate carbofuran (Figures 3.5-3.12). This would be expected since degradation of the ring is the last step in the catabolic pathway (Talebi and Walker 1993). The B and C groups in the soil from Germany stored under laboratory conditions did not differ in their capacity to mineralize carbofuran, whereas they did differ in their capacity to inactivate carbofuran (Figures 3.11). The active microbial population in the soil from Italy was able to mineralize the least µg carbofuan per mg microbial C compared to the other soils (Figure 3.12). As with rates of carbofuran inactivation, no distinction between treatment groups in this soil can be made (Figure 3.12).

When sequential applications of fenamiphos were discontinued for 12 to 16 months, the capacity of a soil to mineralize the pesticide returned to levels observed in previously untreated soils (Anderson and Lafuerza 1992). This allowed reapplication of fenamiphos (Anderson and Lafuerza 1992). However, to avoid the re-development of accelerated microbial degradation a management strategy was developed to alternate fenamiphos applications with applications of another organophosphate nematocide (Anderson and Lafuerza 1992).

Actual degradation rates: carbofuran inactivation

All soils, except the soil from Germany stored under laboratory conditions, showed recovery patterns of carbofuran inactivation over the course of this study (Tables 3.1-3.4).

The carbofuran inactivation rates during the first week were significantly different ($p < 0.05$) in the B group in the soil from New Zealand between the last treatment with technical carbofuran and two months after the last treatment with technical carbofuran (month 0 week 1 = 90.2 ± 5.8%, month 2 week 1 = 69.4 ± 3.4%) (Table 3.1). The carbofuran inactivation rates in the B group for this soil were also significantly different between weeks one and two two months after the last treatment with technical carbofuran (month 2 week 1 = 69.4 ± 3.4%, month 2 week 2 = 91.4 ± 0.7%) (Table 3.1). Regarding the carbofuran inactivation rates of the B group in this soil during the second week, significant differences were observed between the eighth and tenth months after the last treatment with technical carbofuran (month 8 week 2 = 78.4 ± 1.5% , month 10 week 2 = 60.4 ± 5.3%) (Table 3.1). A difference in the carbofuran inactivation rates was observed between weeks two and three 12 months after the last treatment with technical carbofuran (month 12 week 2 = 23.9 ± 2.2%, month 12 week 3 = 72.4 ± 0.7%) (Table 3.1). A significant difference was seen during the third week between the eighth and tenth months after the last treatment with technical carbofuran (month 8 week 3 = 82.9 ± 2.1%, month 10 week 3 = 68.1 ± 1.7%) (Table 3.1). Different carbofuran inactivation rates between weeks three and four were observed 12

months after the last treatment with technical carbofuran (month 12 week 3 = 72.4 ± 0.7%, month 12 week 4 = 80.2 ± 0.8%) (Table 3.1). It is unclear if a recovery pattern can be observed during the fourth week of monitoring across months as the degradation rates appear to oscillate (Table 3.1). The fourth week of monitoring also refers to the actual cumulative degradation rates observed in this study. These results show an initial recovery pattern of carbofuran inactivation occurring during the first week of monitoring as indicated by a lag time, followed sequentially by a similar pattern of lag time during the second and third weeks, respectively.

Similar recovery patterns of carbofuran inactivation were observed in the B group of field soil from Germany. Significant differences of carbofuran inactivation during the first week of monitoring were observed between four and six months after the last treatment with technical carbofuran (month 4 week 1 = 82.6 ± 6.3%, month 6 week 1 = 44.2 ± 9.2%) (Table 3.2). The carbofuran inactivation rates between weeks one and two also differed six months after the last treatment with technical carbofuran (month 6 week 1 = 44.2 ± 9.2%, month 6 week 2 = 75.4 ± 8.2%) (Table 3.2). No other differences within and between weeks were observed (Table 3.2). Therefore, compared to the soil from New Zealand, the field soil from Germany showed a slower overall recovery pattern of carbofuran inactivation.

The recovery pattern of carbofuran inactivation rates in the B group of the soil from Italy was similar to that of the field soil from Germany, except that the B group of the soil from Italy showed even slower recovery (Tables 3.2 and 3.4). A significant difference in carbofuran inactivation during the first week of monitoring did not occur until 16 months after the last treatment with technical carbofuran (month 14 week 1 = 42.1 ± 5.5%, month 16 week 1 = 28.7 ± 0.8%) (Table 3.4). At this time, the difference between the inactivation rates for weeks one and two were also the greatest (month 16 week 1 = 28.7 ± 0.8%, month 16 week 2 = 62.2 ± 5.6%) (Table 3.4). It should be noted that the A group of the soil from Italy had significantly higher inactivation rates over the course of the study compared to the other soils (Table 3.4). This is due in part to abiotic degradation (refer to Chapter 1).

The laboratory soil from Germany did not show signs of recovery (Table 3.3). In particular, both the B and the C groups showed an oscillating pattern of accelerated microbial degradation with almost all of the carbofuran inactivation occurring during the first week of monitoring (Table 3.3). Specifically, the percent of applied ^{14}C-carbonyl carbofuran degraded to $^{14}CO_2$ during the first week of monitoring in both the B and C groups oscillated between approximately 75% and 100% (Table 3.3). Similar oscillating patterns were observed in all C groups which continually showed accelerated degradation rates (Tables 3.1-3.4).

Actual degradation rates: carbofuran mineralization

The soil from New Zealand and the field soil from Germany showed recovery patterns of carbofuran mineralization over the course of this study (Tables 3.5 and 3.6). The recovery patterns of carbofuran mineralization were similar to the recovery patterns of carbofuran inactivation for these soils (Tables 3.1, 3.2, 3.5 and 3.6). For all soils, cumulative mineralization rates were approximately 50% or less than inactivation rates (Tables 3.1-3.8). Again, this would be expected since degradation of the ring is the last step in the catabolic pathway (Talebi and Walker 1993).

The carbofuran mineralization rates during the first week were significantly different ($p < 0.05$) in the B group in the soil from New Zealand between two and four months after the last treatment with technical carbofuran (month 2 week 1 = 34.6 ± 1.3%, month 4 week 1 = 21.0 ± 0.8%) (Table 3.5). The carbofuran mineralization rates between weeks one and two were significantly different two months after the last treatment with technical carbofuran (month 2 week 1 = 34.6 ± 1.3%, month 2 week 2 = 43.5 ± 1.6%) (Table 3.5). Regarding the carbofuran mineralization rates during the second week, significant differences were observed between the tenth and twelfth months after the last treatment with technical carbofuran (month 10 week 2 = 37.2 ± 1.1%, month 12 week 2 = 12.9 ± 2.2%) (Table 3.5). Differences in mineralization rates between weeks two and three were observed 12 months after the last treatment with technical carbofuran (month 12 week 2 = 12.9 ± 2.2, month 12 week 3 = 37.6 ± 0.4%) (Table 3.5).

Lastly, significant differences were observed in mineralization rates measured in the third week between the fourteenth and sixteenth months after the last treatment with technical carbofuran (month 14 week 3 = 36.9 ± 2.0%, month 16 week 3 = 21.4 ± 4.0%) (Table 3.5).

Recovery of carbofuran mineralization rates in the B group of field soil from Germany were evident in the first week of monitoring between four and six months after the last treatment with technical carbofuran (month 4 week 1 = 29.9 ± 5.6%, month 6 week 1 = 14.8 ± 7.3%) (Table 3.6). Differences during the first week of monitoring also occurred between two and four months after the last treatment of carbofuran (Table 3.6). However, the rates four months after the last treatment with technical carbofuran were similar to those for the last treatment of carbofuran and therefore may have been part of an oscillation pattern of accelerated microbial degradation (month 0 week 1 = 31.3 ± 9.3%, month 4 week 1 = 29.9 ± 5.6%) (Table 3.6). For this reason, differences between the first and second week of monitoring are also most evident six months after the last treatment with technical carbofuran (month 6 week 1 = 14.8 ± 7.3, month 6 week 2 = 46.2 ± 4.0%) (Table 3.6). Differences during the second week of monitoring are most apparent between eight and ten months after the last treatment of technical carbofuran (month 8 week 2 = 56.3 ± 0.9%, month 10 week 2 = 44.8 ± 3.0%) (Table 3.6). Carbofuran mineralization during the second week of monitoring reaches the lowest point 14 months after the last treatment with technical carbofuran (month 14 week 2 = 6.8 ± 0.6%), but then increases 16

months after the last treatment with technical carbofuran (month 16 week 2 = 37.3 ± 7.5%) (Table 3.6). The reason for this increase may be partially attributed to changing environmental conditions in the field. Differences between the second and third week of monitoring were evident 12 months after the last treatment with technical carbofuran (month 12 week 2 = 20.2 ± 0.4%, month 12 week 3 = 53.5 ± 0.8%) (Table 3.6). Recovery patterns of carbofuran mineralization in the third and fourth weeks of monitoring were not observed and the rates oscillated, respectively (Table 3.6).

Recovery patterns of carbofuran mineralization were not observed in the soil from Germany stored under laboratory conditions (Table 3.7). Both the B and the C groups in this soil showed an oscillating pattern of accelerated microbial degradation with almost all of the degradation occurring during the first week of monitoring (Table 3.7).

The soil from Italy did not exhibit accelerated rates of carbofuran mineralization at any time, therefore detecting a recovery pattern of carbofuran mineralization was not possible (Table 3.8).

Actual degradation rates: differences between field and laboratory soils

The field soil from Germany showed signs of recovery six months after the last treatment with analytical carbofuran, whereas the soil from Germany stored under laboratory conditions never recovered and exhibited an oscillating pattern

of accelerated microbial degradation rates with most of the degradation occurring during the first week of monitoring (Tables 3.2, 3.3, 3.6 and 3.7). These differences between soils were observed for carbofuran inactivation and mineralization. These results are not in accordance with previous studies that indicate a rapid degradation of carbofuran in the laboratory while carbofuran remained highly active in the field (Suett and Jukes 1990). In the current study, the field soil had a lower carbofuran / soil ratio meaning its overall concentration of carbofuran was lower compared to the soil stored under laboratory conditions. This may account for the recovery patterns observed in the field soil and not in the soil stored under laboratory conditions. In addition, carbofuran could have dissipated in the field plots due to environmental factors such as precipitation. In both soils, the A and C groups showed similar rates of carbofuran inactivation and mineralization, respectively (Tables 3.2, 3.3, 3.6 and 3.7). This indicates both soils responded similarly to carbofuran applications.

Conclusions

In conclusion, no definite correlation could be made between the capacity of these four soils to inactivate and mineralize carbofuran and their actual inactivation and mineralization rates. Hence, studies assessing accelerated microbial degradation should measure actual degradation rates since these methods more accurately resemble the natural condition of soil.

Two major patterns were seen in the four soils in their actual inactivation and mineralization rates. Carbofuran inactivation and mineralization rates were similar for each soil, but the mineralization rates were approximately 50% or lower. Soils that did not show signs of recovery showed oscillating patterns of accelerated microbial degradation with almost all of the degradation occurring within the first week of monitoring. Therefore, the overall patterns must be considered in deciding if a soil shows signs of recovery because statistically significant decreases in degradation rates could be part of an oscillating pattern and not an indication of recovery. Soils that showed an indication of recovery showed a negative sigmoidal curve with variation between weeks across months of monitoring. These soils did not completely return to levels observed in the untreated group.

The field soil from Germany showed signs of recovery in actual carbofuran inactivation and mineralization rates, whereas the soil from Germany stored under laboratory conditions did not. These results suggest laboratory studies may provide conservative estimates of recovery rates in the field. Therefore, future studies should determine the actual dissipation in soil in the field and laboratory and be able to relate the two to ultimately determine the pattern of recovery of accelerated microbial degradation. This would allow the most effective pest management strategy to be developed.

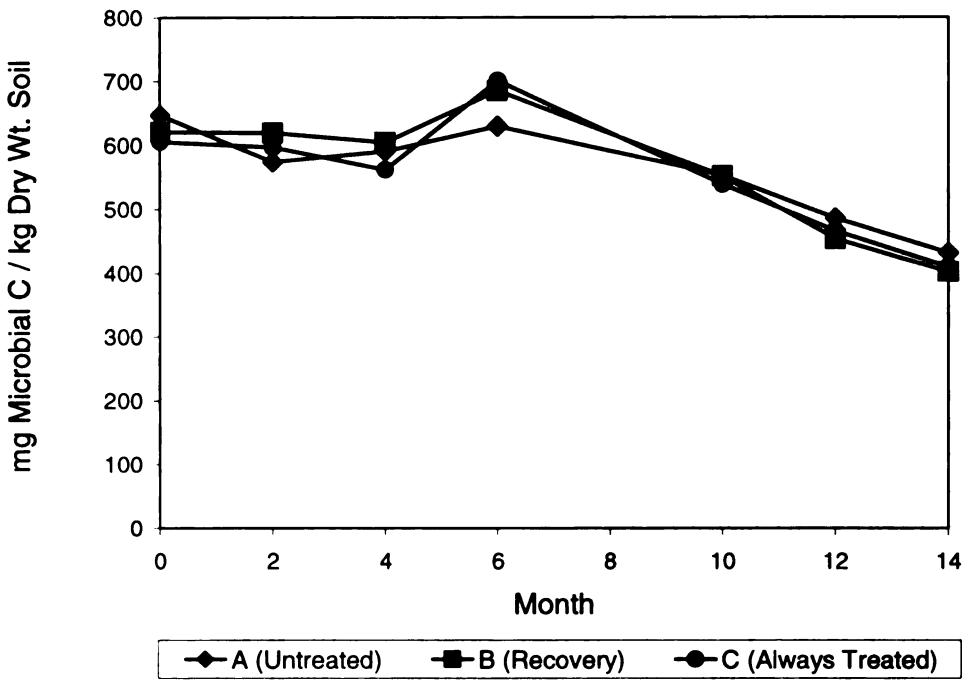

Figure 3.1: Active microbial biomass in the soil from New Zealand. Month 0 marks the last treatment with carbofuran for the recovery group (B).

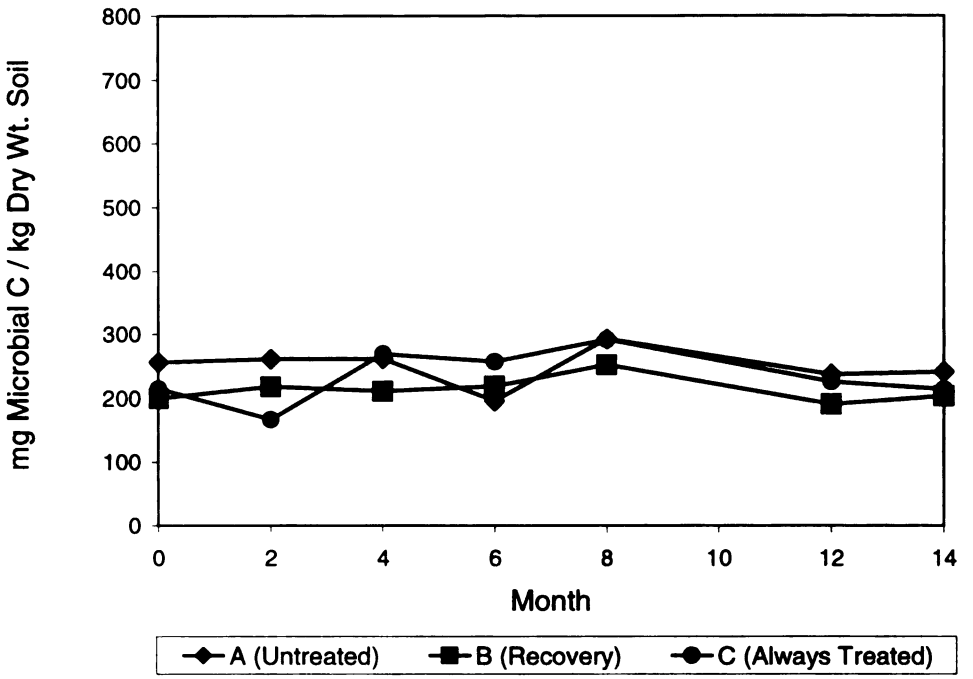

Figure 3.2: Active microbial biomass in the field soil from Germany. Month 0 marks the last treatment with carbofuran for the recovery group (B).

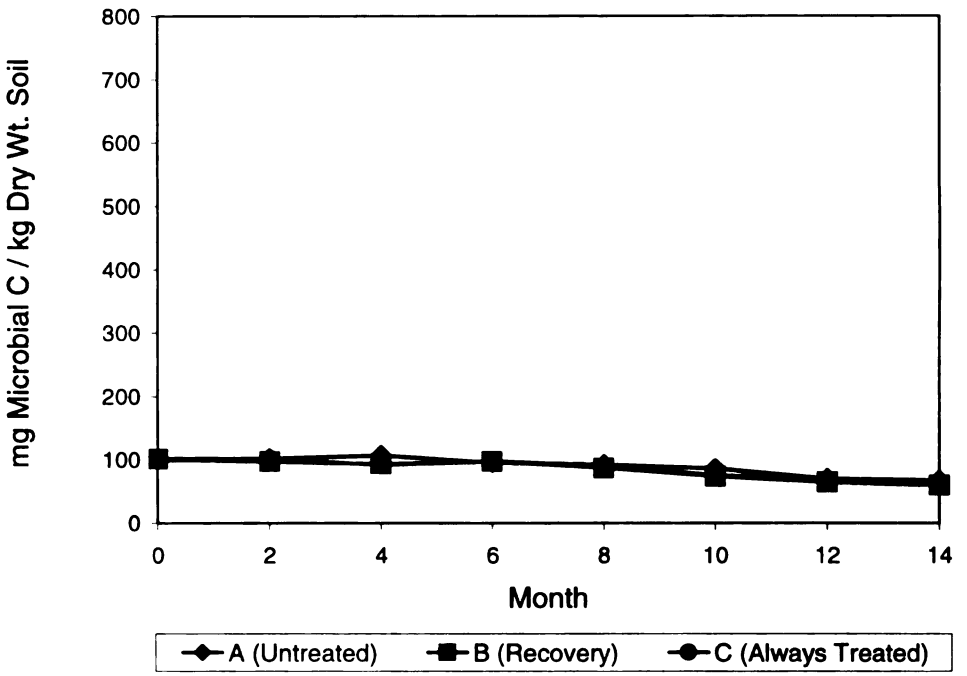

Figure 3.3: Active microbial biomass in the soil from Germany stored under laboratory conditions. Month 0 marks the last treatment with carbofuran for the recovery group (B).

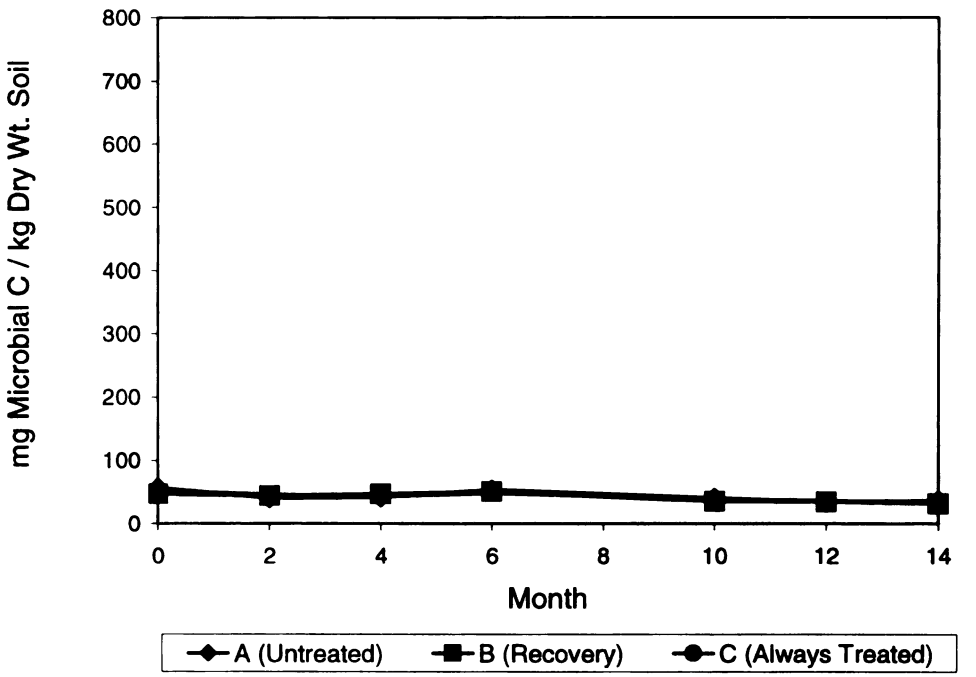

Figure 3.4: Active microbial biomass in the soil from Italy. Month 0 marks the last treatment with carbofuran for the recovery group (B).

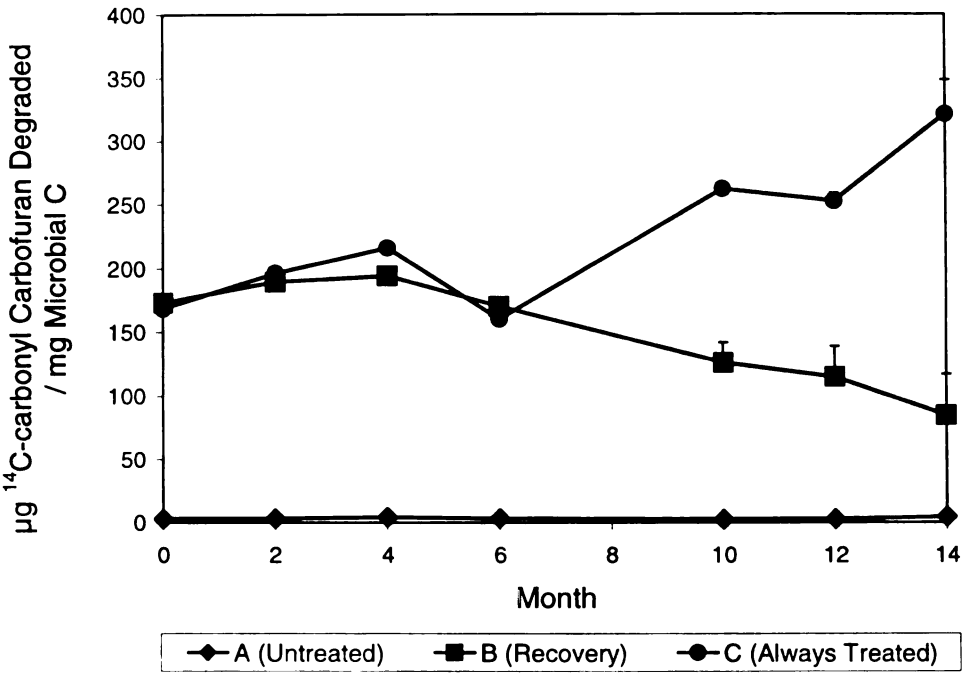

Figure 3.5: The capacity of the soil from New Zealand to inactivate carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

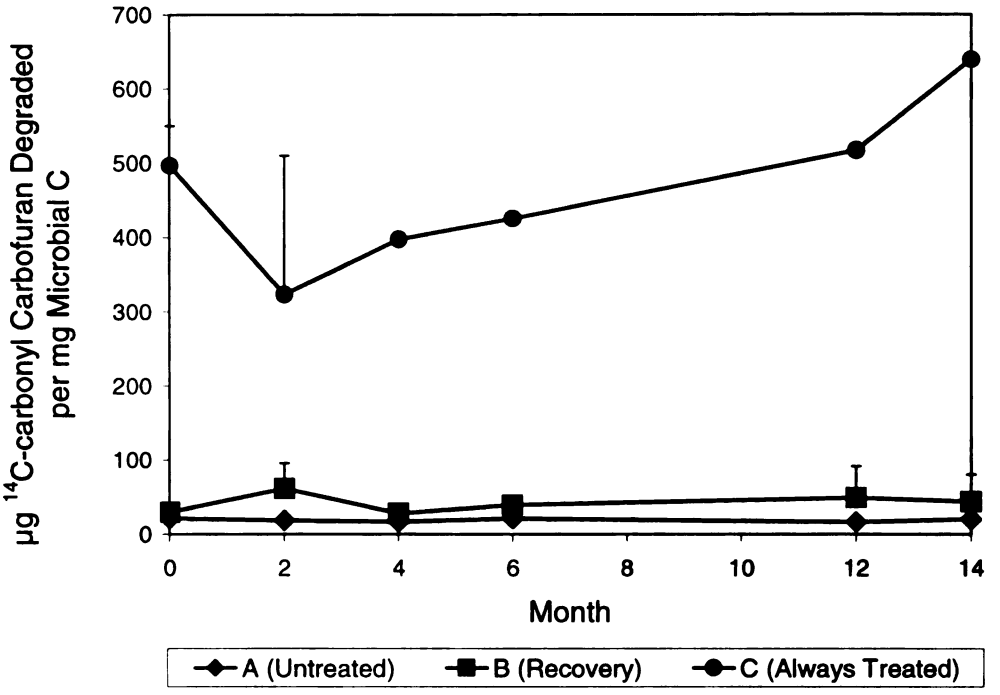

Figure 3.6: The capacity of the field soil from Germany to inactivate carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

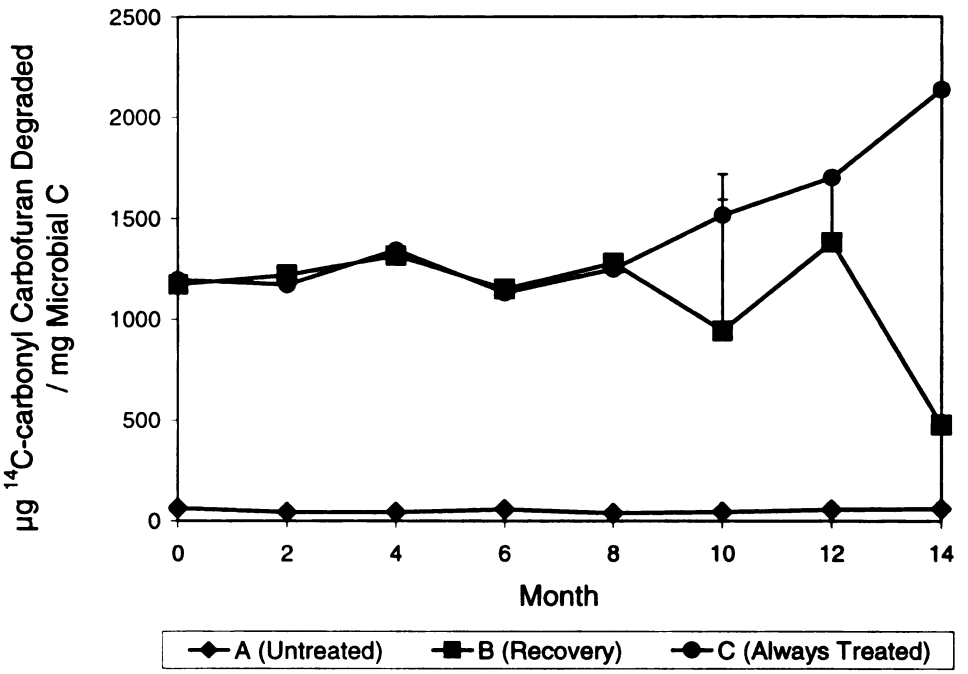

Figure 3.7: The capacity of the soil from Germany stored under laboratory conditions to inactivate carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

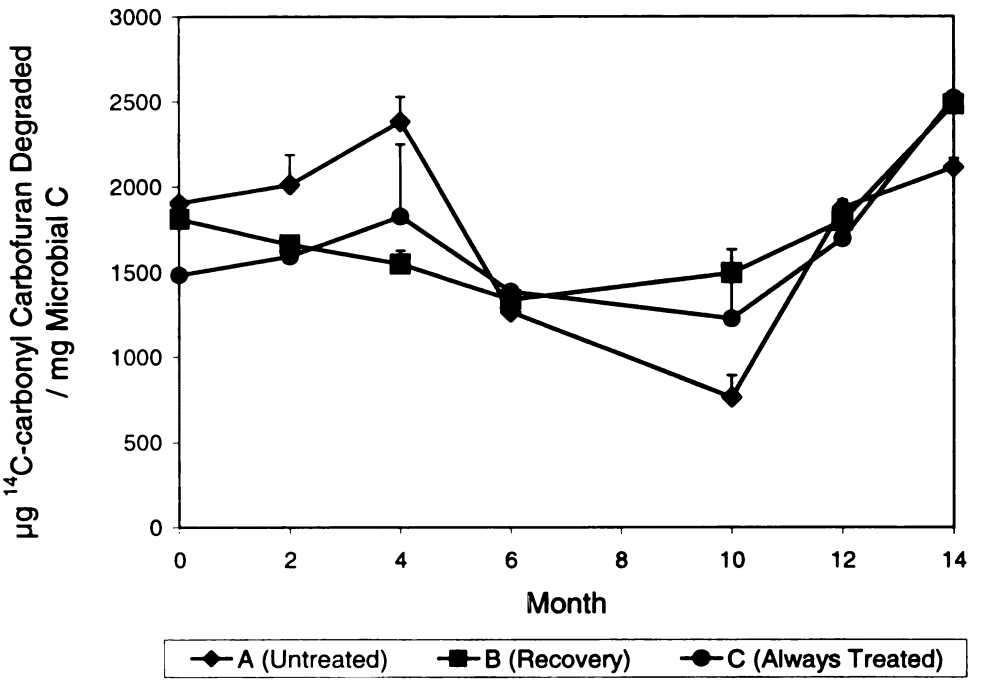

Figure 3.8: The capacity of the soil from Italy to inactivate carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

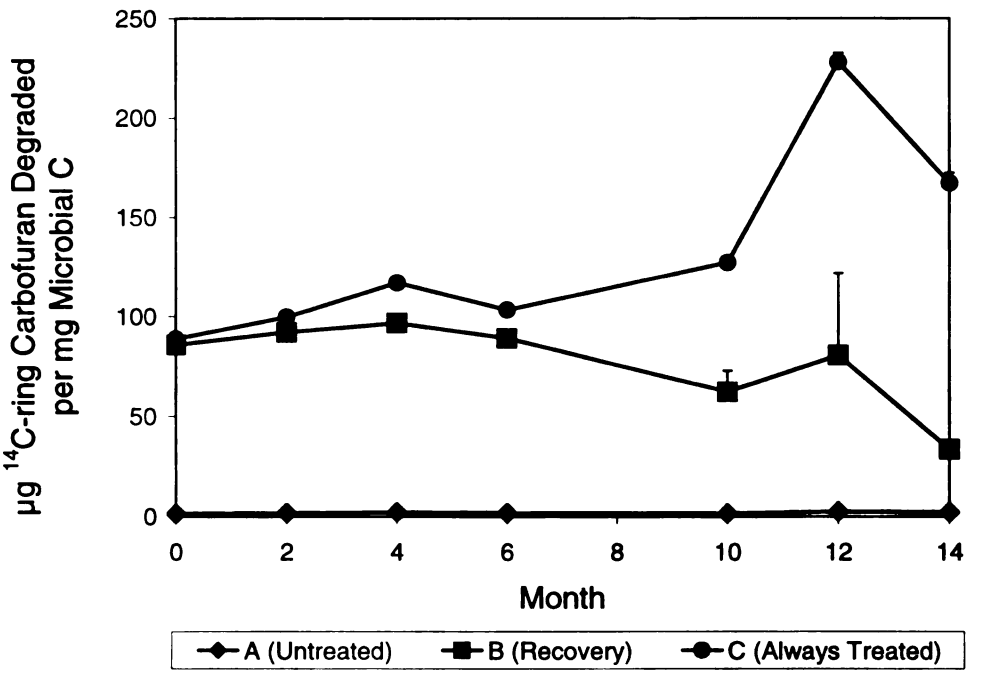

Figure 3.9: The capacity of the soil from New Zealand to mineralize carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

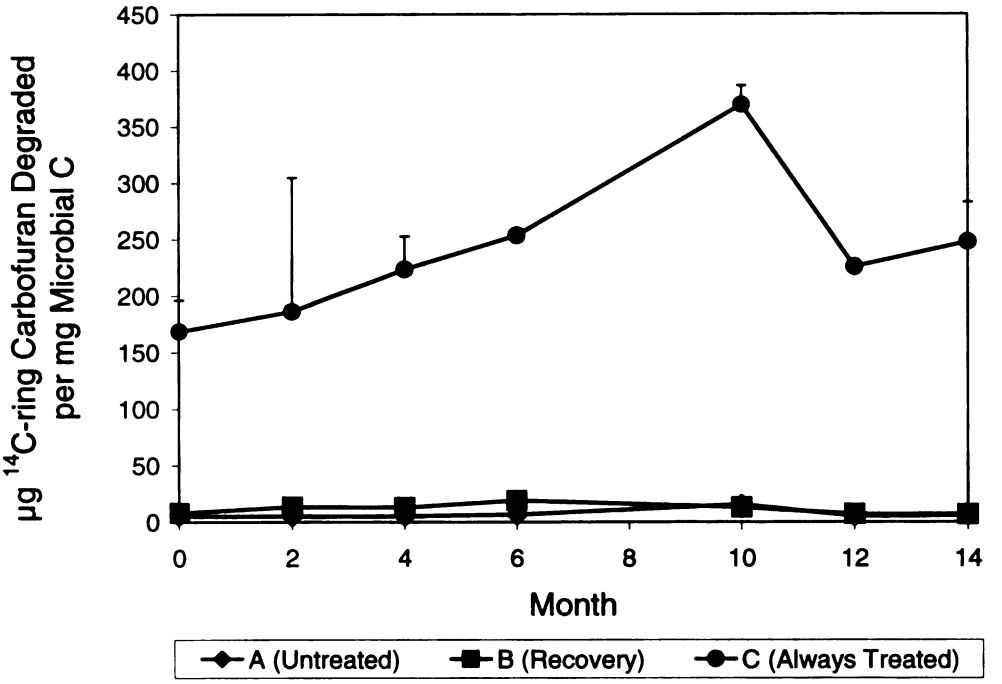

Figure 3.10: The capacity of the field soil from Germany to mineralize carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

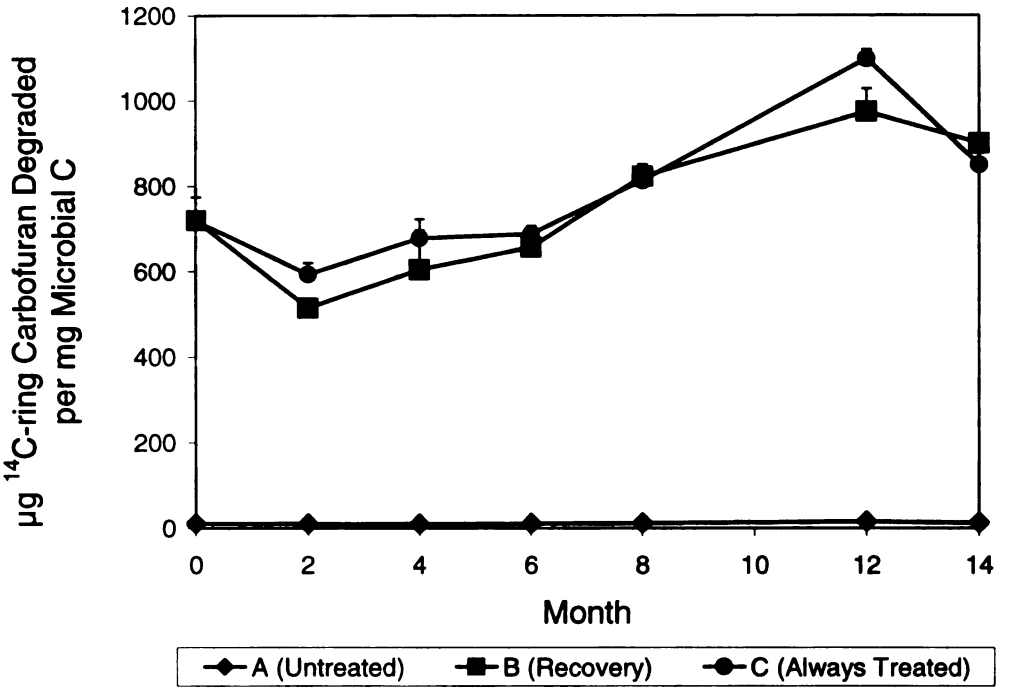

Figure 3.11: The capacity of the soil from Germany stored under laboratory conditions to mineralize carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

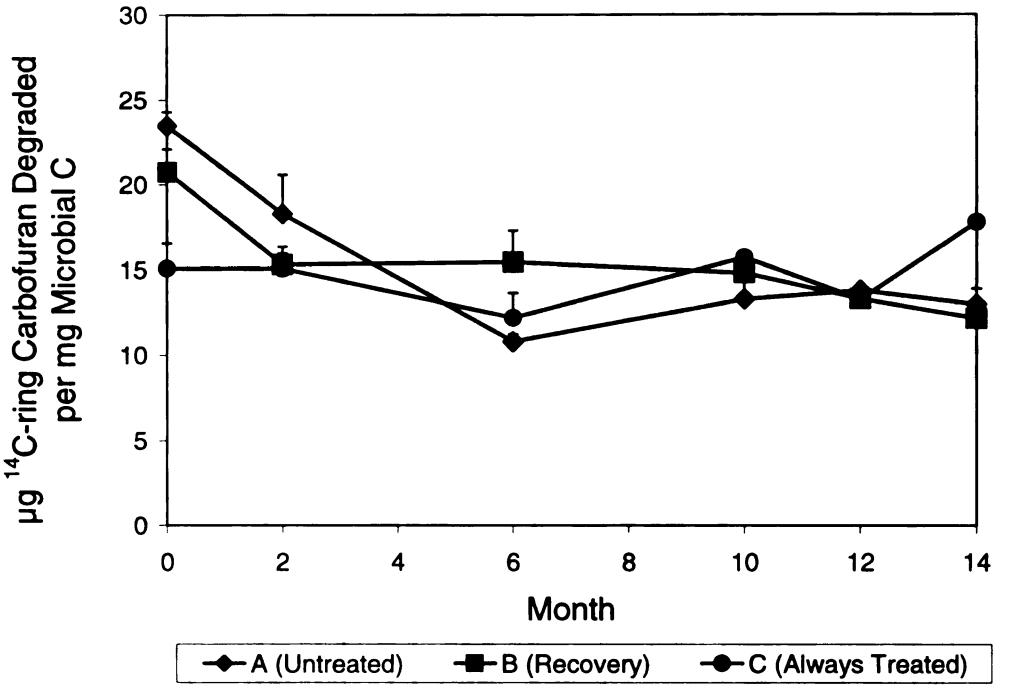

Figure 3.12: The capacity of the soil from Italy to mineralize carbofuran. Month 0 marks the last treatment with carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-carbonyl Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: New Zealand					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	1.25 ± 0.0	2.6 ± 0.1	4.2 ± 0.1	5.8 ± 0.1
	Recovery	90.2 ± 5.8	94.6 ± 6.8	95.7 ± 6.4	96.5 ± 6.3
	Every 2 Months	93.8 ± 1.4	97.5 ± 1.6	98.6 ± 1.7	99.2 ± 1.6
2	None	1.3 ± 0.0	2.8 ± 0.1	4.3 ± 0.2	5.8 ± 0.3
	Recovery	69.4 ± 3.4	81.2 ± 6.7	82.5 ± 6.9	83.7 ± 6.1
	Every 2 Months	91.4 ± 0.7	94.5 ± 0.6	95.4 ± 0.5	95.9 ± 0.5
4	None	1.4 ± 0.0	2.7 ± 0.0	4.0 ± 0.1	5.5 ± 0.1
	Recovery	44.4 ± 5.8	82.6 ± 4.0	84.0 ± 4.6	85.0 ± 4.6
	Every 2 Months	81.9 ± 2.5	83.7 ± 2.5	84.3 ± 2.5	84.7 ± 2.5
6	None	1.1 ± 0.0	2.2 ± 0.0	3.4 ± 0.4	4.4 ± 0.4
	Recovery	12.7 ± 1.3	80.9 ± 3.2	85.8 ± 3.0	86.8 ± 3.1
	Every 2 Months	86.0 ± 3.2	87.9 ± 3.4	88.6 ± 3.5	88.9 ± 3.5
8	None	1.2 ± 0.1	2.5 ± 0.1	4.0 ± 0.1	5.4 ± 0.1
	Recovery	11.5 ± 1.4	78.4 ± 1.5	82.9 ± 2.1	84.2 ± 1.6
	Every 2 Months	95.4 ± 1.8	97.3 ± 2.1	97.9 ± 2.0	98.2 ± 2.0
10	None	1.0 ± 0.0	1.9 ± 0.0	2.9 ± 0.1	3.9 ± 0.1
	Recovery	9.2 ± 2.7	60.4 ± 5.3	68.1 ± 1.7	69.2 ± 1.8
	Every 2 Months	64.9 ± 2.2	65.8 ± 2.7	66.2 ± 2.6	66.5 ± 2.4
12	None	1.1 ± 0.0	2.1 ± 0.1	3.1 ± 0.2	4.2 ± 0.2
	Recovery	2.7 ± 1.5	23.9 ± 2.2	72.4 ± 0.7	80.2 ± 0.8
	Every 2 Months	82.4 ± 2.0	84.5 ± 2.1	85.2 ± 2.2	85.5 ± 2.3
14	None	0.8 ± 0.0	1.8 ± 0.1	2.6 ± 0.1	3.5 ± 0.1
	Recovery	3.3 ± 0.4	30.1 ± 6.3	71.5 ± 3.4	80.3 ± 3.3
	Every 2 Months	76.2 ± 0.3	81.2 ± 0.6	82.8 ± 0.8	84.0 ± 0.9
16	None	0.9 ± 0.0	1.5 ± 0.2	2.2 ± 0.5	3.1 ± 0.6
	Recovery	2.1 ± 0.1	8.7 ± 2.0	32.8 ± 7.4	68.9 ± 5.7
	Every 2 Months	82.0 ± 3.0	87.0 ± 3.1	88.4 ± 3.1	89.2 ± 3.1

Table 3.1: Actual carbofuran inactivation rates in the treatment groups for the soil from New Zealand. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-carbonyl Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: Germany (Field Plot)					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	3.41 ± 0.1	7.6 ± 0.2	12.1 ± 0.4	15.7 ± 0.5
	Recovery	81.7 ± 7.9	98.1 ± 8.6	99.0 ± 8.9	99.5 ± 8.9
	Every 2 Months	98.2 ± 7.3	97.8 ± 7.4	98.3 ± 7.5	99.7 ± 7.5
2	None	2.8 ± 0.0	5.9 ± 0.1	8.9 ± 0.1	11.9 ± 0.2
	Recovery	85.2 ± 3.7	87.3 ± 3.9	87.9 ± 3.9	88.1 ± 3.9
	Every 2 Months	85.5 ± 2.2	89.9 ± 2.0	90.5 ± 1.9	90.8 ± 1.9
4	None	3.0 ± 0.2	6.3 ± 0.2	9.6 ± 0.2	12.7 ± 0.2
	Recovery	82.6 ± 6.3	83.0 ± 7.4	83.8 ± 7.4	84.2 ± 7.4
	Every 2 Months	87.5 ± 0.1	88.9 ± 0.1	89.4 ± 0.1	89.7 ± 0.1
6	None	2.0 ± 0.2	5.2 ± 0.3	7.5 ± 0.7	10.6 ± 0.6
	Recovery	44.2 ± 9.2	75.4 ± 8.2	76.0 ± 8.1	76.4 ± 8.2
	Every 2 Months	73.2 ± 9.3	74.8 ± 9.3	75.1 ± 9.3	75.3 ± 9.3
8	None	3.0 ± 0.3	7.1 ± 0.8	9.9 ± 2.3	13.5 ± 2.4
	Recovery	27.4 ± 1.2	78.9 ± 2.0	79.9 ± 2.2	80.4 ± 2.3
	Every 2 Months	85.1 ± 1.3	86.8 ± 1.9	87.6 ± 1.5	88.2 ± 1.1
10	None	3.2 ± 0.0	7.1 ± 0.3	10.4 ± 1.2	13.7 ± 1.6
	Recovery	55.4 ± 5.7	71.4 ± 1.1	74.1 ± 3.3	74.8 ± 4.0
	Every 2 Months	72.4 ± 2.1	73.9 ± 2.2	74.4 ± 2.2	74.7 ± 2.2
12	None	3.5 ± 0.1	7.1 ± 0.1	10.4 ± 0.2	13.1 ± 0.2
	Recovery	4.7 ± 1.3	70.7 ± 8.8	85.0 ± 1.3	85.8 ± 1.2
	Every 2 Months	88.9 ± 0.8	90.6 ± 0.9	91.1 ± 1.0	91.4 ± 1.0
14	None	3.8 ± 0.3	7.3 ± 0.3	10.8 ± 0.4	14.3 ± 0.4
	Recovery	4.6 ± 1.0	79.9 ± 0.9	84.2 ± 2.2	86.1 ± 1.8
	Every 2 Months	82.3 ± 2.2	85.2 ± 2.8	86.1 ± 3.1	86.5 ± 3.1
16	None	3.5 ± 0.1	7.1 ± 0.2	10.3 ± 0.2	13.2 ± 0.2
	Recovery	4.7 ± 1.6	65.7 ± 6.7	76.7 ± 3.9	78.7 ± 4.9
	Every 2 Months	74.5 ± 3.3	80.7 ± 3.6	82.6 ± 3.8	83.4 ± 3.8

Table 3.2: Actual carbofuran inactivation rates in the treatment groups for the field soil from Germany. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-carbonyl Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: Germany (Laboratory)					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	2.50 ± 0.0	4.5 ± 0.1	6.5 ± 0.1	8.3 ± 0.2
	Recovery	83.1 ± 5.5	83.8 ± 5.5	84.1 ± 5.6	84.2 ± 5.6
	Every 2 Months	87.7 ± 1.3	88.4 ± 1.4	88.7 ± 1.4	88.9 ± 1.4
2	None	1.7 ± 0.0	3.7 ± 0.0	5.7 ± 0.1	7.8 ± 0.3
	Recovery	83.2 ± 1.7	84.0 ± 1.6	84.2 ± 1.6	84.4 ± 1.6
	Every 2 Months	92.8 ± 4.5	93.6 ± 4.6	93.9 ± 4.6	94.1 ± 4.6
4	None	1.7 ± 0.0	3.6 ± 0.1	5.7 ± 0.0	7.7 ± 0.1
	Recovery	89.1 ± 4.1	90.0 ± 4.2	90.4 ± 4.3	90.7 ± 4.3
	Every 2 Months	92.7 ± 7.4	93.5 ± 7.6	93.9 ± 7.6	94.0 ± 7.6
6	None	1.6 ± 0.0	3.3 ± 0.0	5.1 ± 0.1	6.9 ± 0.1
	Recovery	97.8 ± 1.2	99.2 ± 1.5	99.7 ± 1.5	99.9 ± 1.5
	Every 2 Months	88.6 ± 3.0	89.4 ± 3.1	89.8 ± 3.1	90.0 ± 3.1
8	None	1.6 ± 0.1	3.6 ± 0.1	5.7 ± 0.1	7.8 ± 0.1
	Recovery	88.1 ± 5.3	89.0 ± 5.2	89.4 ± 5.3	89.6 ± 5.5
	Every 2 Months	91.8 ± 5.6	92.4 ± 5.6	92.8 ± 5.7	93.0 ± 5.7
10	None	1.6 ± 0.0	3.4 ± 0.0	5.2 ± 0.0	6.9 ± 0.0
	Recovery	76.8 ± 3.8	77.6 ± 4.0	77.9 ± 4.1	78.1 ± 4.1
	Every 2 Months	80.5 ± 2.0	81.1 ± 1.9	81.4 ± 2.0	81.6 ± 2.0
12	None	1.4 ± 0.0	4.1 ± 0.0	5.8 ± 0.1	7.5 ± 0.1
	Recovery	84.2 ± 5.8	84.5 ± 5.8	84.9 ± 5.8	85.1 ± 5.8
	Every 2 Months	83.0 ± 0.4	83.7 ± 0.4	84.0 ± 0.4	84.2 ± 0.3
14	None	2.2 ± 0.2	4.3 ± 0.2	6.4 ± 0.4	8.9 ± 0.7
	Recovery	87.4 ± 3.7	89.7 ± 3.9	90.1 ± 3.9	90.4 ± 4.0
	Every 2 Months	84.6 ± 3.3	85.4 ± 3.5	85.7 ± 3.5	85.9 ± 3.5
16	None	1.1 ± 0.0	2.6 ± 0.1	4.0 ± 0.1	5.5 ± 0.2
	Recovery	76.4 ± 3.3	83.4 ± 7.0	84.1 ± 7.0	84.4 ± 7.0
	Every 2 Months	84.6 ± 3.6	86.4 ± 3.2	86.7 ± 3.3	86.9 ± 3.4

Table 3.3: Actual carbofuran inactivation rates in the treatment groups for the soil from Germany stored under laboratory conditions. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-carbonyl Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: Italy					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	17.40 ± 3.2	34.8 ± 5.5	46.0 ± 1.9	54.5 ± 2.2
	Recovery	67.7 ± 3.0	80.2 ± 1.7	84.1 ± 2.3	86.5 ± 3.0
	Every 2 Months	72.4 ± 8.7	86.2 ± 3.6	88.8 ± 3.8	89.8 ± 4.6
2	None	27.1 ± 3.3	51.5 ± 5.8	68.2 ± 7.2	78.8 ± 8.0
	Recovery	55.4 ± 9.3	69.3 ± 8.6	73.6 ± 8.4	75.9 ± 8.2
	Every 2 Months	61.9 ± 3.9	71.5 ± 3.3	74.6 ± 3.9	76.2 ± 4.3
4	None	27.3 ± 3.5	51.9 ± 5.6	66.2 ± 8.4	75.5 ± 8.9
	Recovery	63.2 ± 3.6	83.6 ± 4.6	88.8 ± 5.3	91.1 ± 5.4
	Every 2 Months	67.1 ± 5.6	83.8 ± 6.5	88.9 ± 5.8	91.4 ± 5.6
6	None	23.3 ± 0.6	43.5 ± 1.2	57.4 ± 1.2	67.7 ± 1.1
	Recovery	65.3 ± 5.6	78.4 ± 6.2	82.3 ± 6.0	84.7 ± 5.5
	Every 2 Months	61.3 ± 0.5	71.1 ± 0.3	74.4 ± 0.3	76.4 ± 0.2
8	None	27.8 ± 1.2	48.9 ± 1.5	62.3 ± 1.4	70.0 ± 2.3
	Recovery	55.4 ± 10.2	74.7 ± 0.4	79.5 ± 0.3	80.9 ± 0.9
	Every 2 Months	63.1 ± 3.6	75.4 ± 2.8	79.5 ± 2.3	82.0 ± 2.1
10	None	22.4 ± 4.9	39.8 ± 7.7	47.1 ± 8.1	52.2 ± 9.1
	Recovery	43.1 ± 3.4	49.7 ± 0.3	51.6 ± 0.1	52.5 ± 0.6
	Every 2 Months	48.3 ± 1.3	58.4 ± 4.8	61.5 ± 5.6	63.2 ± 6.3
12	None	25.2 ± 3.6	45.7 ± 6.1	58.5 ± 6.8	66.1 ± 7.5
	Recovery	46.0 ± 9.5	63.6 ± 8.3	68.4 ± 7.2	71.0 ± 6.6
	Every 2 Months	58.4 ± 7.9	67.9 ± 7.8	70.7 ± 7.6	72.3 ± 7.7
14	None	23.1 ± 3.2	42.3 ± 5.0	56.1 ± 5.5	62.7 ± 6.2
	Recovery	42.1 ± 5.5	71.0 ± 6.8	76.3 ± 7.5	77.9 ± 7.9
	Every 2 Months	53.9 ± 8.8	64.3 ± 9.8	68.9 ± 9.3	70.1 ± 9.0
16	None	27.8 ± 3.2	50.2 ± 5.4	63.6 ± 6.3	71.7 ± 6.5
	Recovery	28.7 ± 0.8	62.2 ± 5.6	70.7 ± 4.0	74.1 ± 3.3
	Every 2 Months	48.8 ± 2.9	67.3 ± 1.4	72.6 ± 1.6	75.6 ± 2.5

Table 3.4: Actual carbofuran inactivation rates in the treatment groups for the soil from Italy. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-ring Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: New Zealand					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	0.47 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	1.5 ± 0.1
	Recovery	39.4 ± 1.1	42.6 ± 2.1	44.4 ± 2.8	45.9 ± 2.6
	Every 2 Months	40.8 ± 0.4	44.8 ± 0.4	46.9 ± 0.4	47.7 ± 0.8
2	None	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	1.6 ± 0.0
	Recovery	34.6 ± 1.3	43.5 ± 1.6	45.3 ± 2.6	46.7 ± 2.7
	Every 2 Months	44.7 ± 2.1	48.3 ± 2.4	50.0 ± 2.7	51.2 ± 2.7
4	None	0.6 ± 0.0	0.8 ± 0.0	1.2 ± 0.1	1.5 ± 0.1
	Recovery	21.0 ± 0.8	44.1 ± 1.4	46.5 ± 1.5	48.1 ± 1.6
	Every 2 Months	49.0 ± 1.1	52.1 ± 1.6	53.6 ± 1.7	54.8 ± 1.8
6	None	0.5 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	1.4 ± 0.1
	Recovery	6.2 ± 0.3	41.7 ± 2.5	45.4 ± 3.1	46.0 ± 3.2
	Every 2 Months	47.9 ± 2.4	50.8 ± 2.5	52.4 ± 2.6	53.3 ± 2.7
8	None	0.5 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.6 ± 0.1
	Recovery	5.7 ± 0.8	41.9 ± 2.1	45.4 ± 0.5	47.2 ± 0.4
	Every 2 Months	47.0 ± 4.0	49.3 ± 4.1	50.3 ± 4.7	51.1 ± 5.0
10	None	0.6 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.5 ± 0.1
	Recovery	4.2 ± 0.3	37.2 ± 1.1	41.2 ± 3.4	42.5 ± 4.2
	Every 2 Months	43.8 ± 1.6	46.0 ± 1.8	46.7 ± 1.3	47.2 ± 1.0
12	None	0.5 ± 0.0	0.7 ± 0.0	1.0 ± 0.0	1.2 ± 0.1
	Recovery	1.3 ± 0.1	12.9 ± 2.2	37.6 ± 0.4	42.4 ± 1.2
	Every 2 Months	40.9 ± 0.9	43.7 ± 0.9	45.0 ± 1.0	45.9 ± 1.0
14	None	0.5 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	1.3 ± 0.0
	Recovery	1.3 ± 0.1	14.4 ± 3.2	36.9 ± 2.0	43.4 ± 1.7
	Every 2 Months	42.5 ± 2.0	46.3 ± 2.3	47.8 ± 2.4	49.0 ± 2.5
16	None	0.5 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	1.3 ± 0.0
	Recovery	0.9 ± 0.1	4.9 ± 1.2	21.4 ± 4.0	42.3 ± 1.6
	Every 2 Months	47.5 ± 1.6	51.9 ± 2.3	53.5 ± 2.4	54.7 ± 2.6

Table 3.5: Actual carbofuran mineralization rates in the treatment groups for the soil from New Zealand. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-ring Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: Germany (Field Plot)					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	0.78 ± 0.0	1.5 ± 0.0	2.4 ± 0.0	3.2 ± 0.1
	Recovery	31.3 ± 9.3	50.7 ± 3.5	54.4 ± 3.3	56.1 ± 3.5
	Every 2 Months	37.6 ± 2.0	46.2 ± 2.6	50.3 ± 2.8	52.1 ± 2.9
2	None	0.7 ± 0.0	1.4 ± 0.1	2.3 ± 0.6	3.4 ± 1.4
	Recovery	42.6 ± 0.9	51.5 ± 1.0	54.0 ± 1.8	55.5 ± 2.3
	Every 2 Months	40.2 ± 2.1	52.8 ± 1.5	56.1 ± 1.6	58.0 ± 1.6
4	None	0.8 ± 0.0	1.4 ± 0.0	2.2 ± 0.1	3.2 ± 0.3
	Recovery	29.9 ± 5.6	49.9 ± 2.6	54.2 ± 2.9	56.2 ± 3.1
	Every 2 Months	38.3 ± 1.9	45.5 ± 2.1	48.5 ± 2.3	50.2 ± 2.4
6	None	0.6 ± 0.1	1.1 ± 0.0	1.5 ± 0.3	1.9 ± 0.5
	Recovery	14.8 ± 7.3	46.2 ± 4.0	49.0 ± 4.5	51.1 ± 4.7
	Every 2 Months	27.2 ± 2.6	33.9 ± 2.3	36.0 ± 2.3	37.1 ± 1.8
8	None	0.8 ± 0.1	1.6 ± 0.1	2.5 ± 0.2	3.5 ± 0.2
	Recovery	8.9 ± 5.7	56.3 ± 0.9	60.4 ± 0.9	62.8 ± 0.9
	Every 2 Months	29.7 ± 0.8	35.9 ± 1.4	38.0 ± 1.3	39.7 ± 1.4
10	None	0.7 ± 0.0	1.5 ± 0.1	2.2 ± 0.1	3.1 ± 0.1
	Recovery	1.6 ± 0.1	44.8 ± 3.0	48.6 ± 4.0	50.5 ± 4.4
	Every 2 Months	27.2 ± 1.6	33.7 ± 1.9	36.1 ± 2.1	37.7 ± 2.1
12	None	0.9 ± 0.0	1.6 ± 0.0	2.3 ± 0.0	3.1 ± 0.0
	Recovery	1.0 ± 0.1	20.2 ± 0.4	53.5 ± 0.8	56.5 ± 1.8
	Every 2 Months	32.7 ± 0.3	39.5 ± 0.4	42.7 ± 0.2	44.4 ± 0.2
14	None	0.9 ± 0.1	1.7 ± 0.0	2.6 ± 0.1	3.6 ± 0.1
	Recovery	0.9 ± 0.0	6.8 ± 0.6	40.2 ± 1.8	54.3 ± 2.0
	Every 2 Months	31.4 ± 0.6	36.2 ± 0.5	39.4 ± 0.7	41.5 ± 0.8
16	None	0.9 ± 0.0	1.7 ± 0.1	2.6 ± 0.1	3.4 ± 0.2
	Recovery	1.2 ± 0.3	37.3 ± 7.5	48.4 ± 0.4	51.0 ± 1.0
	Every 2 Months	26.0 ± 0.6	31.8 ± 1.0	34.3 ± 1.2	35.6 ± 1.3

Table 3.6: Actual carbofuran mineralization rates in the treatment groups for the field soil from Germany. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-ring Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: Germany (Laboratory)					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	0.59 ± 0.01	0.9 ± 0.0	1.3 ± 0.0	1.6 ± 0.0
	Recovery	34.2 ± 1.85	38.1 ± 2.0	40.3 ± 2.2	41.6 ± 2.3
	Every 2 Months	32.4 ± 1.37	36.2 ± 1.5	38.1 ± 1.7	39.3 ± 1.7
2	None	0.5 ± 0.0	0.8 ± 0.1	1.0 ± 0.2	1.4 ± 0.3
	Recovery	15.8 ± 10.1	18.2 ± 11.6	19.8 ± 12.6	20.1 ± 12.6
	Every 2 Months	30.6 ± 3.2	34.5 ± 3.5	36.9 ± 3.6	38.5 ± 3.8
4	None	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	1.5 ± 0.0
	Recovery	20.6 ± 1.1	23.7 ± 1.4	26.2 ± 1.5	27.8 ± 1.5
	Every 2 Months	29.4 ± 1.6	32.9 ± 1.7	35.5 ± 1.8	37.1 ± 1.9
6	None	0.5 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.5 ± 0.0
	Recovery	21.0 ± 2.0	23.9 ± 3.0	26.1 ± 3.5	27.8 ± 3.5
	Every 2 Months	26.2 ± 1.3	29.3 ± 1.4	31.4 ± 1.5	32.5 ± 2.1
8	None	0.5 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	1.5 ± 0.0
	Recovery	19.7 ± 0.9	23.1 ± 1.0	24.8 ± 0.7	26.1 ± 1.2
	Every 2 Months	24.3 ± 0.9	27.4 ± 1.2	29.4 ± 1.3	30.8 ± 1.4
10	None	0.5 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	1.5 ± 0.1
	Recovery	17.0 ± 1.2	19.6 ± 1.5	21.6 ± 1.6	22.9 ± 1.7
	Every 2 Months	19.6 ± 0.6	22.8 ± 0.8	24.7 ± 0.9	25.6 ± 1.2
12	None	0.5 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	1.4 ± 0.0
	Recovery	20.9 ± 1.6	25.0 ± 1.7	27.2 ± 1.8	28.7 ± 1.7
	Every 2 Months	19.7 ± 0.5	23.2 ± 0.5	25.3 ± 0.5	26.6 ± 0.5
14	None	0.5 ± 0.0	0.8 ± 0.0	1.1 ± 0.0	1.4 ± 0.0
	Recovery	14.6 ± 0.8	17.3 ± 1.0	18.9 ± 1.0	20.4 ± 1.1
	Every 2 Months	11.9 ± 0.8	13.6 ± 1.0	15.1 ± 1.1	16.4 ± 1.2
16	None	0.5 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	1.3 ± 0.0
	Recovery	11.6 ± 0.6	16.7 ± 1.8	18.6 ± 2.0	19.8 ± 2.1
	Every 2 Months	16.8 ± 2.4	19.9 ± 2.9	21.8 ± 3.2	23.1 ± 3.3

Table 3.7: Actual carbofuran mineralization rates in the treatment groups for the soil from Germany stored under laboratory conditions. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

Cumulative % Applied ^{14}C-ring Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks Soil: Italy					
Month	**Treatment**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
0	None	0.55 ± 0.0	1.1 ± 0.0	1.8 ± 0.0	2.5 ± 0.1
	Recovery	0.84 ± 0.0	1.6 ± 0.0	2.3 ± 0.1	3.1 ± 0.1
	Every 2 Months	0.81 ± 0.0	1.5 ± 0.1	2.3 ± 0.1	3.0 ± 0.1
2	None	0.6 ± 0.0	1.3 ± 0.1	2.1 ± 0.1	3.0 ± 0.1
	Recovery	0.8 ± 0.0	1.5 ± 0.1	2.1 ± 0.4	2.7 ± 0.8
	Every 2 Months	0.9 ± 0.0	1.7 ± 0.0	2.2 ± 0.2	3.0 ± 0.3
4	None	0.6 ± 0.1	1.3 ± 0.1	1.9 ± 0.2	2.5 ± 0.4
	Recovery	0.8 ± 0.0	1.7 ± 0.1	2.5 ± 0.1	3.4 ± 0.1
	Every 2 Months	0.8 ± 0.0	1.3 ± 0.3	1.7 ± 0.6	2.3 ± 0.9
6	None	0.5 ± 0.0	1.1 ± 0.1	1.7 ± 0.0	2.4 ± 0.0
	Recovery	0.7 ± 0.0	1.2 ± 0.3	1.9 ± 0.3	2.7 ± 0.3
	Every 2 Months	0.8 ± 0.0	1.3 ± 0.3	1.6 ± 0.4	2.0 ± 0.5
8	None	0.6 ± 0.0	1.2 ± 0.3	1.8 ± 0.7	2.4 ± 1.0
	Recovery	0.8 ± 0.0	1.4 ± 0.4	2.0 ± 0.7	2.8 ± 0.8
	Every 2 Months	0.8 ± 0.1	1.6 ± 0.2	2.4 ± 0.2	3.2 ± 0.3
10	None	0.6 ± 0.0	1.3 ± 0.0	2.1 ± 0.0	2.8 ± 0.1
	Recovery	0.7 ± 0.0	1.5 ± 0.0	2.0 ± 0.3	2.5 ± 0.5
	Every 2 Months	0.8 ± 0.0	1.6 ± 0.0	2.2 ± 0.3	2.6 ± 0.5
12	None	0.5 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	2.2 ± 0.1
	Recovery	0.6 ± 0.0	1.2 ± 0.0	1.8 ± 0.0	2.4 ± 0.0
	Every 2 Months	0.6 ± 0.0	1.3 ± 0.1	1.9 ± 0.1	2.6 ± 0.2
14	None	0.5 ± 0.0	1.1 ± 0.0	1.8 ± 0.1	2.4 ± 0.1
	Recovery	0.6 ± 0.0	1.3 ± 0.1	2.1 ± 0.1	2.6 ± 0.1
	Every 2 Months	0.7 ± 0.0	1.5 ± 0.0	2.3 ± 0.0	2.8 ± 0.0
16	None	0.4 ± 0.0	1.0 ± 0.0	1.6 ± 0.1	2.3 ± 0.2
	Recovery	0.5 ± 0.0	1.2 ± 0.1	1.8 ± 0.1	2.4 ± 0.1
	Every 2 Months	0.6 ± 0.0	1.3 ± 0.1	2.0 ± 0.1	2.7 ± 0.0

Table 3.8: Actual carbofuran mineralization rates in the treatment groups for the soil from Italy. Month 0 marks the last treatment of technical carbofuran for the recovery group (B).

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CHAPTER IV

DOES CARBOFURAN APPLICATION AND DEVELOPMENT OF ACCELERATED MICROBIAL DEGRADATION AFFECT SOIL BACTERIAL COMMUNITY STRUCTURE?

Abstract

Accelerated microbial degradation is an agricultural problem that arises when pesticides are repeatedly applied to the same soil resulting in reduced efficacy or failure of the compound. The objective of this study was to determine possible changes in bacterial community structure in soils with varying degradation rates of carbofuran. Four soils, each with three different treatment levels, were used. Two of these soils have the same field origin, but a portion had been stored under laboratory conditions and developed different rates of degradation. 16S rDNA was extracted from each soil and treatment group, and community "fingerprints" were obtained using a terminal restriction fragment length polymorphism (T-RFLP) analysis with restriction endonucleases *HhaI*, *HaeIII* and *MspI*. Histograms of individual fragments indicate application of carbofuran did not result in shifts in bacterial communities between treatments within soils. These results indicate development of accelerated microbial degradation of carbofuran in the soils studied is probably caused by an increase of enzyme activity of microorganisms or to a population change below the level of sensitivity of T-RFLP. Cluster analysis of individual fragments indicated that treatments within soils grouped most closely, and the soil stored under laboratory conditions

was most similar to the same soil in field plots. To date, few studies have been conducted to assess the effects of pesticides on soil microbial community structure using novel methods in molecular biology. These studies are important in understanding how accelerated microbial degradation of pesticides develops as well as potential effects of pesticides on ecosystem processes. Combining novel methods in molecular biology with traditional methods also provides a more accurate measure of potential stress of pesticides on microbial communities. This would be a beneficial approach to include in mandatory risk assessment tests to provide more insight as to the possible ecological implications of changes in bacterial diversity on soil fertility.

Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum, systemic insecticide and nematocide used to control a range of pests including corn rootworm (*Diabrotica* spp., Felsot et al. 1981), cabbage root fly (*Phorbia* spp. Karpouzas et al. 2001) and carrot fly (*Psila* spp. Suett 1986) in a variety of agricultural crops such as rice, corn and beets (Morel-Chevillet et al. 1996). Accelerated degradation of carbofuran is a well documented problem and occurs when soil is repeatedly exposed to the pesticide (Felsot et al. 1981). Accelerated degradation of carbofuran and carbamothioate herbicides has also been correlated with an increased ability of soil microorganisms to use the compounds as growth substrates (Williams et al. 1976, Felsot et al. 1981, Harris et al. 1984, Camper 1987, Moorman 1988, Mueller et al. 1989). Usually,

carbofuran degrading microorganisms use the methylamine for growth (Desaint et al. 2000, Topp et al. 1993, Feng et al. 1997, Parekh et al. 1995).

Two major hypotheses exist as to how accelerated degradation develops. The most widely accepted hypothesis is an increase in the number of microorganisms capable of degrading the chemical (Trabue et al. 2001). In laboratory studies, accelerated degradation rates of carbofuran were correlated with significant increases in total bacterial populations (Edwards et al. 1992). In a field study, two annual applications of carbofuran resulted in an increase of the microbial community capable of degrading the ^{14}C-carbonyl carbofuran (Trabue et al. 2001). Similar results have been reported for 2,4-D (Holben et al. 1992). The second hypothesis is accelerated microbial degradation results from an increase in the enzyme activity, but not from an increase in community size (Trabue et al. 2001). Three successive annual carbofuran applications in the field did not result in an increase in the number of the soil microorganisms capable of mineralizing the ^{14}C-UL-carbofuran, which suggests degradation of the ring structure is a cometabolic process, i.e. increase in enzyme activity (Trabue et al. 2001). Different enzymes which hydrolyze N-methylcarbamate compounds have been purified and characterized and further studies with these are needed (Hashimoto et al. 2002, Tomasek and Karns 1989, Chapalamadugu and Chaudhry 1992).

Evidence supporting these two hypotheses indicates due to the variety of bacteria and enzymes associated with carbofuran degradation it can be expected

that treatments with this compound would have an impact on the microbial community structure (Morel-Chevillet et al. 1996). In particular, the development of accelerated microbial degradation is generally thought to result from physiological adaptation of native species and not because of species shifts in the community or increased populations of adapted species (Moorman 1988). The persistence of accelerated microbial degradation may be due to a persistent pesticide-induced competitive advantage that may even be in a very small segment of the total microbial community (Edwards et al. 1992).

The widespread use of pesticides in agriculture is of increasing concern (Johnsen et al. 2001). The major concern is their potential toxicity to non-target organisms (Crecchio et al. 2001). Although most pesticides on the market today are low-dose, great uncertainty regarding their influence on the soil microbial community exists (Crecchio et al. 2001). For example, much remains unknown about their effects on the abundance and composition of soil microorganisms, which may strongly influence soil fertility (Crecchio et al. 2001).

Current registration procedures for pesticides require the evaluation of the effect of pesticides on the environment, specifically testing for non-target effects on a single species or on microbial communities (Engelen et al. 1998). The impact of pesticides on soil microbial communities for registration purposes is tested in view of their role in sustaining biogeochemical cycles and their functions in supporting plant growth (Engelen et al. 1998). Successful registration of a

pesticide is contingent on the absence of effects on processes that are believed to be important for soil fertility (Johnsen et al. 2001). In the European Union, studies of soil sensitivity required for the registration of pesticides are usually conducted using worst case scenarios (Lynch 1995).

Internationally, there is much debate which laboratory tests should be mandatory in the registration process to study the effects of pesticides on soil microorganisms (Engelen et al. 1998). Tests required in Germany include substrate induced respiration (SIR), dehydrogenase activity and nitrogen turnover (Anderson et al. 1990). Methods used in risk assessments like SIR measure the metabolic behavior of a range of microorganisms which are not equally sensitive to pesticide application and it is therefore difficult to accurately interpret the real meaning of respiration rate variations (Vallaeys et al. 1997). Process level measurements such as total number of microorganisms, respiration rates and enzyme activities may be insensitive to changes in microbial structure due to the redundancy of these functions (Ogram et al. 1995). In other words, current tests for pesticide registration may indicate how the entire community responds to potentially toxic interactions but may be considered to be limited in their ecological significance (Engelen et al. 1998). Therefore, implementation of current guidelines for the approval of pesticides may not detect changes in microbial diversity which also affect soil fertility (Johnsen et al. 2001).

Some international agreements such as the Agenda 21, a United Nations initiative, aim to conserve biological diversity (www.un.org). Agenda 21 calls for urgent and decisive action to conserve and maintain genes, species and ecosystems and supports the international Convention on Biological Diversity (www.un.org). Two of its specific objectives are 1) "to promote broader international and regional cooperation in furthering scientific and economic understanding of the importance of biodiversity and its functions in ecosystems" and 2) "to develop measures and arrangements to implement the rights of countries of origin of genetic resources...to benefit from the biotechnological development and the commercial utilization of products derived from such resources" (www.un.org). This is particularly important since the degree of soil microbial diversity is still largely unknown, which suggests many more useful products, e.g. pharmaceuticals, are yet to be identified (Rondon et al. 1999). More research about microbial diversity is needed to describe and protect these genetic resources for the preservation of natural ecosystems and the future benefit of mankind (Rondon et al. 1999).

Pesticide effects on microbial diversity should be also be studied because diversity is an indication of how stressed the ecosystem has been and a decrease in diversity would result in a decrease of the biological system to respond to perturbations (Johnsen et al. 2001). The potential stress due to pesticide application is determined by both the chemical characteristics of the pesticide and the abundance of native microbial community (Jones and

Ananyeva 2001). Since species abundance and diversity measurements are sensitive indicators of environmental conditions, evaluating changes in community structure can be used to study its response to stress (Atlas 1984 a and b). Genetic diversity in microorganisms reflects the total genetic potential of the community and because of selective growth and successions it also reflects changes in environmental conditions (Atlas 1984). Species richness (the number of species within a community) and species evenness (the sizes of species populations within a community) are essential parameters in defining community structure and diversity (Liu et al. 1997). Meaningful studies of microbial communities consider the abundance and distribution of species as well as the functional diversity and redundancy in the community (Kent and Triplett 2002).

Traditional methods used to study the effects of pesticides on soil microbial communities include cultivation and isolation of microbial species (Sommerville and Greaves 1987). However, the fraction of cells that may be cultured from environmental samples is not representative of the abundance or diversity of the microbial community present in the environment (Kent and Triplett 2002). To overcome bias of culture-dependent methods, the use of molecular methods is gaining importance due to their detection of microorganisms in their natural habitats (El Fantroussi et al. 1999). Molecular methods therefore provide a wider picture of diversity than culture-dependent methods (Johnsen et al. 2001).

Torsvik et al. (1990) were the first to estimate the number of prokaryotic genomes in soil using culture-independent methods. By comparing the reassociation time of total community DNA with a standard curve of reassociation kinetics of a known number of cultured genomes, Torsvik et al. estimated 4000 distinct genomes per gram of soil (1990). The complexity of information in nucleic acid molecules most significantly describes community structure and diversity (Engelen et al. 1998). Moreover, genetic diversity in bacteria can be defined as the amount and distribution of genetic information in an assemblage or a community (Atlas 1984).

Although novel methods in molecular biology are gaining popularity, they have not been substantially used to study effects of pesticides on microbial diversity (Johnsen et al. 2001). Few studies to date have been published that use culture-independent methods to assess potential effects of pesticides on soil bacterial diversity (Kent and Triplett 2002). Of the nucleic acid molecules, 16S rRNA and 16S rDNA are best suited to gain a more detailed view of community structure and to determine the abundance of phylogenetically meaningful sequences (Engelen et al. 1998). Community "fingerprint" methods using 16S rRNA and 16S rDNA show the frequency distribution of different sequences in environmental samples and can be used to study changes in communities impacts on species diversity due to induced conditions such as pesticide application (Engelen et al. 1998). These methods include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE),

amplified rDNA restriction analysis (ARDRA), and terminal-restriction fragment length polymorphism (T-RFLP) (Crecchio et al. 2001).

T-RFLP is a powerful tool that can be used to rapidly assess the diversity of complex bacterial communities (Liu et al. 1997). The T-RFLP pattern serves as a community "fingerprint" and is a composite of the number of fragments with unique lengths and the relative abundance of each fragment is reflected by the size of each peak in the electropherogram (Liu et al. 1997). This analysis yields quantitative data about each T-RF detected, including size in base pairs and peak height (intensity of fluorescence) (Blackwood et al. 2003). T-RFLP can be used to assess changes in the microbial community structure on different temporal and spatial scales as well as from environmental perturbations. T-RFLP has three major advantages compared to other "fingerprinting" methods (Marsh 1999). The first is that all terminal fragment sizes that result from digestion of a PCR product can be directly compared with terminal fragments in growing sequence databases and hence phylogenetic inference can be made (Marsh 1999). The second advantage is that nucleic acid sequencing technology has significantly higher resolution than other electrophoretic systems (Marsh 1999). Lastly, results from T-RFLP gel analysis is immediate and the output is digital (Marsh 1999). In addition, the T-RFLP method has been shown to be a very robust method in analyzing community profiles. Minimal variability has been observed between replicate environmental samples as a result of replicate restriction digestions, PCR amplifications and replicate DNA extractions (Osborn

et al. 2000, Ayala-del-Río 2002). In one study, the percentages of variation in complex samples only ranged from 3.3% to 6.2% (Ayala-del-Río 2002). Hence, the results of T-RFLP analyses are reproducible and environmental samples have been shown to be homogenous and representative of the microbial community (Osborn et al. 2000, Ayala-del-Río 2002, Braker et al. 2001, Marsh 1999).

The overall objective of this study was to use T-RFLP to investigate changes in soil microbial communities due to carbofuran application thereby exploring its suitability to be possibly included in future risk assessment studies to evaluate the potential impact of pesticides on genetic diversity. Our hypotheses were 1) the response of soil microbial communities depends on the structure of the community and prior exposure of the community to carbofuran and 2) treatments with carbofuran select species in the native community that are more fit/better adapted for using carbofuran as a substrate and that diversity would subsequently decrease as certain species were enriched and became dominant.

Materials and Methods

Pesticide

Pure analytical (chemical purity > 99%) and radiochemical (radiochemical purity > 99%) 2,2-dimethyl-2,3-dihydrobenzo-furanyl-7-methylcarbamate (carbofuran) were supplied by Bayer CropScience (Monheim, Germany). Stock and treating solutions are described in Chapter 2.

Soils

Three soils from Germany, Italy and New Zealand were used in this study. Soils from Italy and New Zealand were shipped to the Bayer Research Center in Monheim, Germany and stored under laboratory conditions. Part of the soil from Germany was stored under laboratory conditions, and part was analyzed under field conditions. Specific soil characteristics and handling procedures are presented in Chapter 2. Briefly, each soil was divided into three treatment groups: never treated with carbofuran (A), treated every two months with analytical carbofuran with treatments discontinuing after for six months (B-recovery), and treated continually every two months with analytical carbofuran (C). To determine the effects of treatments with analytical carbofuran, degradation rates of ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran to $^{14}CO_2$ in moist soil were determined over a four week period just prior to these treatments. Degradation rates of soils at the time of the current study are presented in Chapter 3 (month 10). At this time, degradation rates of both ^{14}C-carbonyl carbofuran and ^{14}C-ring carbofuran significantly decreased in soils from New Zealand and Germany (field plot), indicating recovery of these soils had begun.

DNA extraction

Total DNA was extracted directly from 0.8 g dry wt. soil using the FastDNA® SPIN kit for Soil (BIO101, Carlsbad, CA) according to the manufacturer's instructions, including a bead-beating step performed with a vortexer. The DNA

was quantified and analyzed spectrophotometrically by taking measurements at 260 nm.

PCR conditions

PCR amplifications of 16S rDNA from total soil DNA extracts were performed with a total volume of 50 µl in a model 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). One µl of DNA template was amplified in reaction mixtures containing 0.2 µM each of primers 8-27F (Giovannoni 1991) and 1392-1407R (Amann et al. 1995), 3mM $MgCl_2$, 0.2mM each of deoxyribonucleotide triphosphate, 0.2 mg/ml bovine serum albumin, 1X PCR buffer, and 2.5U *Taq* polymerase. After a denaturation step of 3 min at 95°C, amplification reactions were performed with 30 cycles of denaturation (45 s, 94°C), primer annealing (1 min, 57°C) and primer extension (2 min, 72°C) and a final extension step of 7 min at 72°C. PCR products had a fluorescent tag of primer 8F that was HEX-labeled to ensure that only terminal fragments were measured.

Products of four replicate PCR's for each sample were combined and concentrated using a speedvac (Savant, Holbrook, NY) for approximately 45 min until a final volume of 50 µl was reached. The PCR products were analyzed by electrophoresis on 1% TAE agarose gels followed by staining with ethidium bromide (0.5 mg $liter^{-1}$). Bands were visualized by UV excitation and purified using a QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions.

16S rRNA T-RFLP

Hydrolysis was performed with three different restriction endonucleases in digestions with a single tetrameric enzyme each. These were *HaeIII* (GG'CC), *HhaI* (GCG'C) and *MspI* (C'CGG), where the prime shows the site of cleavage (Braker et al. 2001 and Liu et al. 1997). Each digestion (15 µl), containing approximately 100 ng of purified PCR product plus 10 U of restriction endonucleases *HaeIII, HhaI* or *MspI* was cleaved overnight at 37°C with in the manufacturer's recommended reaction buffers (New England BioLabs, Beverly, MA). To prevent the internal standard from cleavage, the restriction endonucleases were deactivated by heating the reaction mixture to 65°C for 25 min after the reaction was completed. Since the presence of ions can interfere with the uptake of DNA using electrokinetic injection in capillary electrophoresis due to preferential injection of higher charge-to-mass molecules, each inactivated restriction digest was diluted with water (Sigma, St. Louis, MO) to a final volume of 500 µl and desalted using Microcon columns (Millipore, Bedford, MA). The digests were then concentrated in a speedvac (Savant) to yield final volumes of 25 µl. Approximately one third of the desalted restriction digest was loaded onto the capillary electrophoresis system 3100 Genetic Analyzers (PE Biosystems, Foster City, CA). The injection time was 30 s and the injection voltage was 3 kV. After electrophoresis, the lengths of fluorescently labeled terminal restriction fragments (T-RF's) were analyzed by comparison with the internal standard

using GeneScan 3.1 (PE Applied Biosystems, Foster City, CA) and Genotyper 2.0 (PE Applied Biosystems) software.

Analysis of T-RFLP's

For each sample, peaks greater than 50 units above background fluorescence were analyzed by manually aligning fragments to the size standard. Manual alignment of peaks was based primarily on the size of the peaks, although the pattern of peaks was also used to determine their alignment when groups of overlapping peaks were found between samples. To prevent detection of primers and uncertainties of size determination, terminal fragments smaller than 50 bp and larger than 900 bp were not used in the analysis. Communities were characterized by the number and heights of the peaks. All samples were simultaneously aligned for each restriction enzyme. The relative abundance of T-RF's within the sections was determined by calculating the ratio between the peak height of each peak and the total peak height of all peaks within one sample. Ratios were converted to percentages, and peaks > 1% of the total are displayed as histograms. These images in this dissertation are presented in color. T-RFLP's were also analyzed by the presence or absence of T-RF's by calculating dendograms based on a 1/0 matrix (1 presence; 0 absence of a given T-RF) using Euclidean and Pearson distances and Gene Cluster 3.0 software (Eisen et al. 1998). Lengths of predominant bacterial 16S rDNA T-RF's were theoretically compared to those of aligned sequences using the TAP T-RFLP

function of the Ribosomal Database Project program Beta2, release 7.1 (http://www.rdp.com.edu).

Results and Discussion

Overall change in community structure

Although significant differences in degradation rates of ^{14}C-carbonyl and ^{14}C-ring carbofuran existed between non-enhanced and enhanced soils (Chapter 2) no apparent shifts in the bacterial community structure were observed in any of the soils (Figures 4.1 and 4.2). These results are not entirely surprising. Due to the numerous reports of accelerated degradation of carbofuran, it is generally accepted that carbofuran is not toxic to most soil microorganisms and there is little or no effect of carbofuran on soil microbial communities when it is applied at field application rates. Using traditional methods, i.e. isolation techniques and mineralization rates of carbon and nitrogen, Das and Mukherjee (2000) found carbofuran did not seem to affect the distribution of the two most dominant bacteria, *Bacillus* spp. and *Micrococcus* spp., in soil. A dominant fungus in soil, *Aspergillus* spp. was also unaffected by carbofuran treatments (Das and Mukherjee 2000).

Studies with other pesticides have yielded similar results. For example, the fungicide fenpropimorph did not affect the bacterial diversity on young barley roots using colony counts and rDNA DGGE "fingerprinting" (Thirup et al. 2001). Using RAPD "fingerprints", no changes in bacterial community structure in

response to 2,4-D application were seen in agricultural soils (Xia et al. 1995, Ogram et al. 1995). In studies with propanil and prometryne, DGGE and ARDRA analyses showed that neither the varying concentrations of the compound or incubation time resulted in an apparent change in the corresponding community profiles, although HPLC indicated that propanil almost completely disappeared from soil samples (Crecchio et al. 2001). Moreover, soil enrichment cultures with the phenyl urea herbicides diuron and chlorotoluron as the sole sources of carbon and nitrogen showed no difference in the treated and control groups in the degradation rates of these compounds (El Fantroussi et al. 1999).

The fact that these pesticides were degraded but no changes in microbial community structure were observed seems to indicate accelerated degradation developed in these soils due to an increase in the enzyme activity or due to a population change below the level of sensitivity of T-RFLP. For example, it is possible that species responsible for the accelerated degradation rates responds positively to the presence of the pesticide, although individual growth may be small relative to the entire microbial community (Edwards et al. 1992). Adding carbofuran at a rate of 10 μl g^{-1} soil has been found to support a bacterial mass of 1.8 x 10^6 cells g^{-1} soil (Trabue 1997). Since the total bacterial population in agricultural soils is approximately 1 x 10^8 cells g^{-1} soil, changes in the community structure due to carbofuran additions would occur in less than 1 % of the total community and therefore go undetected.

Some studies, however, seem to support the hypothesis that the development of accelerated microbial degradation is due to an increase in the number of microorganisms capable of degrading the chemical. Using traditional isolation methods, application of insecticides belonging to the chlorinated hydrocarbon, organophosphate, carbamate and synthetic pyrethroid groups in general were found to significantly increase the population of bacteria, actinomycetes and fungi in soil (Das and Mukherjee 2000). One 2,4-D study that combined traditional methods, an MPN assay, and molecular methods, hybridization probes, detected a dramatic response of the microbial community with the initial treatment of 2,4-D, and the population that predominated after the first treatment was not succeeded or displaced with further treatments (Holben et al. 1992). Moreover, using culture-independent techniques, both the structure and metabolic potential of the soil microbial communities were found to be significantly affected by a 10-year history of urea herbicide application (El Fantroussi et al. 1999). TGGE patterns, which represent species abundance, showed that exposure to dinoterb resulted in a significant decrease of a variety of species which was compensated for by an increased abundance in, or a novel appearance of other species (Engelen et al. 1998). Using DGGE, long-term application of the herbicides atrazine and metolachlor significantly decreased the biodiversity of type I methanotrophs in agricultural soils, but the diversity of type II methanotrophs was slightly higher in the treated soil (Seghers et al. 2001).

Phylogenetic determination with RDP

Due to the high level of diversity found within T-RFLP's it was not possible to assign bacterial species to T-RF's using the TAP T-RFLP tool of the Ribosomal Database Project. Other studies have also indicated the complexity of T-RFLP profiles obtained from environmental samples precluded phylogenetic determination of individual fragments (Braker et al. 2001, Kitts 2001, Liu et al. 1997, Marsh 1999, Ayala-del-Río 2002) Obtaining specific phylogenic information from results of T-RFLP analyses by comparing T-RF's to databases may be more applicable to microcosms with a few, known strains than for environmental samples (Liu et al. 1997, Konstantinidis et al. 2003). However, gaining phylogenetic information from T-RFLP also includes the assumption that the current databases accurately reflect the distribution patterns of natural communities (Marsh 1999). In order to gain more specific phylogenetic information, future studies should include constructing a clone library from the amplified phylogenetic markers to better assess the microbial community structure (Kent and Triplett 2002). Clone libraries are a fine-scale approach for taxonomic assignment of dominant community members (Kent and Triplett 2002) and are also useful in identifying and characterizing novel species (Kitts 2001).

Specific changes in community structures

Electropherograms for all samples digested with *HhaI* are shown in Figure 4.1. T-RFLP's were compared by calculating the relative abundances of individual T-RF's within samples (Figures 4.1 and 4.2). The histogram shown represents

peaks >1% of the total T-RF's of digests with *HhaI* (Figure 4.2). All restriction endonucleases yielded similar results (data not shown). Relatively high species diversity, represented as total number of peaks, was observed in all samples. The average total number of peaks for treatments within a soil using *HhaI* were: 56 ± 1 for soil from Germany stored under laboratory conditions, 71 ± 3 for the soil from the field plots in Germany, 54 ± 2 for the soil from Italy and 46 ± 2 for the soil from New Zealand. The total peak heights of all fragments can be compared between samples and represents the species evenness. The average of the total peak heights of the treatments within soils in terms of fluorescent units were: 46196 ± 3240 for the soil from Germany stored under laboratory conditions, 53285 ± 9534 for the soil from the field plots in Germany, 23996 ± 4937 for the soil from Italy and 25156 ± 5958 for the soil from New Zealand.

Only the most abundant species of very complex communities will contribute to a T-RF pattern thereby underestimating diversity (Engelen et al. 1998). In addition, universal primers by definition should be complementary to all know sequences, however primers that are designed by using existing 16S rRNA information in databases may represent only a fraction of the total species diversity of the microbial world (Amann et al. 1995, Torsvik et al. 1990). Type II errors, i.e. not detecting differences between community profiles when they actually are different, can also exist in T-RFLP analyses thereby further underestimating diversity (Blackwood et al. 2003). Therefore, the histogram represents >>1% of total community.

Of the number of total number of fragments (109) in all samples digested with *HhaI,* 26 (24% of total fragments) were found in all soils and treatment groups. These fragments had sizes of: 50, 54, 55, 57, 61, 65, 77, 79, 85, 89, 97, 125, 150, 191, 193, 204, 212, 217, 232, 235, 341, 347, 355, 364, 513 and 548. Thirty (28% of total fragments) fragments were unique to one soil: 2 (2% of total fragments) in the soil from Germany stored under laboratory conditions, 11 (10% of total fragments) in the soil from the field plot in Germany, 6 (6% of total fragments) in the soil from Italy and 11 (10% of total fragments) in the soil from New Zealand.

Cluster analyses

Cluster analyses are suitable for exploratory data analysis and summarize as much variability in the data set as possible within a dendrogram (Krzanowski and Marriott 1994). Cluster analyses showed that treatments within a soil grouped closely together (Figure 4.3). Therefore, the different soils were reliably differentiated using the T-RFLP analysis. Between soils, the soil from Germany stored under laboratory conditions and the same soil from the field plot in Germany were most similar (Figure 4.3). Results shown are for *HhaI* only, but all restriction endonucleases yielded similar results. Dendograms calculated with Euclidean and Pearson distances did not differ, indicating both distance calculations were appropriate and sensitive and that variability within samples was minimal. Including just those peaks that were > 1% of total did not result in

improvement of clustering (data not shown). This has also been reported by Blackwood et al. (2003).

Conclusions

To date, few studies have used culture-independent techniques to assess potential changes in microbial community due to pesticide application (Kent and Triplett 2002, Johnsen et al. 2001). Results from the few studies that have been published report varying impacts of pesticides on microbial communities. An interpretation of these results in terms of correlating causal mechanisms and the impacts of pesticides remains speculative.

More studies using genetic "fingerprint"-based methods are needed and they can be a valid complement and/or alternative to biochemical and microbiological methods used to study microbial community structure (Crecchio et al. 2001). Targeting specific members of a community, e.g. organisms important in C and N mineralization, may be a valuable compliment to improve the sensitivity of these methods to detect changes in these groups. Combining novel methods in molecular biology with traditional methods would allow comparison of methods and also contribute to a better understanding of the traditional methods (Johnsen et al. 2001). For example, the bacterial community structure in soil may be significantly affected by pesticide use even though the overall metabolic parameters such as C and N mineralization appear unaffected (Johnsen et al. 2001). Future studies should focus on using novel methods in conjunction with

traditional methods to ensure the most accurate assessment of effects of pesticides on the soil microbial community can be made and included in mandatory risk assessment studies. Future risk assessment tests requiring molecular methods which complement traditional studies would provide a more accurate measure of potential stress of pesticides on microbial communities. Specifically, these studies could be used to analyze microorganisms important in C and N mineralization and to possibly identify new indicator or key organisms in biogeochemical cycling (Johnsen et al. 2001). This would provide more insight as to the possible ecological implications of changes in bacterial diversity on soil fertility (Johnsen et al. 2001).

Taken one step further, measurements of the effects of pesticides on microbial diversity should also take temporal and spatial heterogeneity into account (Johnsen et al. 2001). The effect of pesticides on the soil microbial community may initially be non-significant but may cause successions over time which lead to altered microbial activities (Johnsen et al. 2001). Studies mandatory for risk assessments should therefore incorporate a way of measuring microbial diversity when the pesticide is applied to see potential immediate toxic effects as well as long-term effects on processes due to successions in the microbial community (Johnsen et al. 2001).

In conclusion, describing "healthy" microbial communities in soil, including their natural variability to assess potential negative impacts of pesticides still remains

a scientific challenge (Engelen et al. 1998). More studies are needed that monitor population dynamics within communities in response to experimental treatment and/or changing environmental conditions.

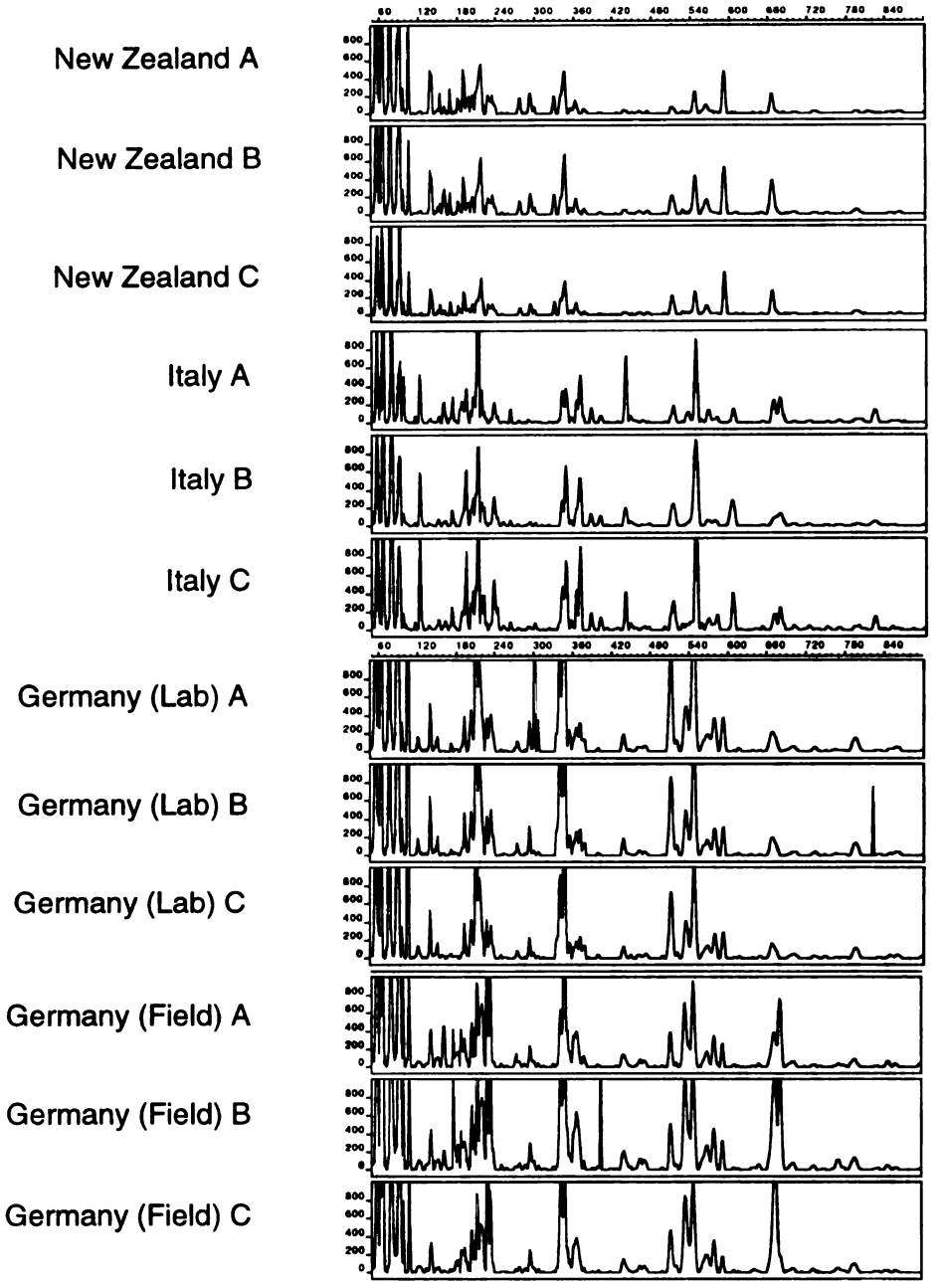

Figure 4.1: T-RFLP electropherograms of PCR amplified 16S rDNA bacterial genes from soil samples after digestion with *Hhal*. Each soil was divided into three treatment groups: never treated with carbofuran (A), treated every two months with analytical carbofuran with treatments discontinuing after for six months (B-recovery), and treated continually every two months with analytical carbofuran (C).

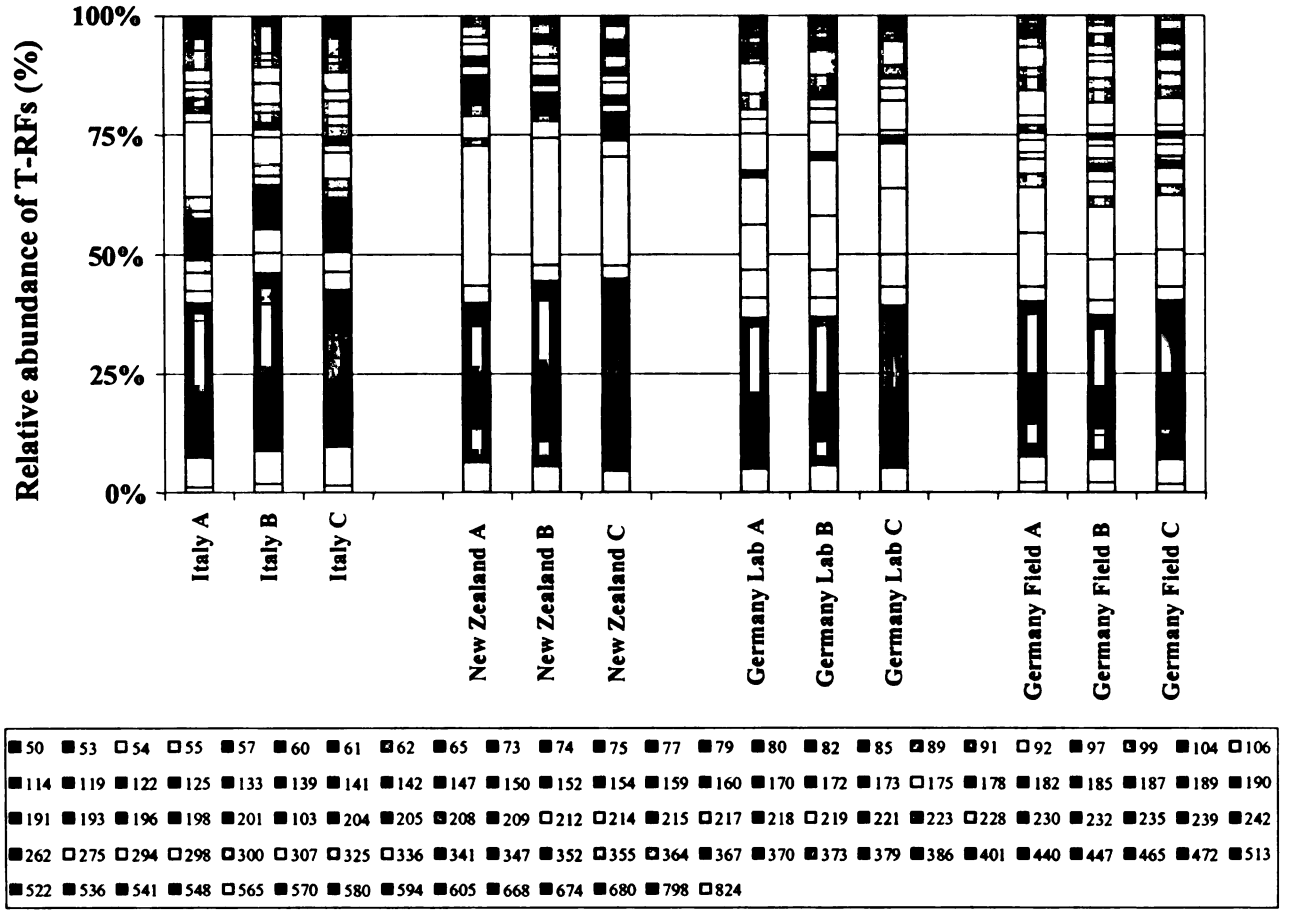

Figure 4.2: Relative abundance of Bacterial T-RF's > 1% from soil samples after digestion with *HhaI*. Each soil was divided into three treatment groups: never treated with carbofuran (A), treated every two months with analytical carbofuran with treatments discontinuing after for six months (B-recovery), and treated continually every two months with analytical carbofuran (C). Numbers in the legend indicate the sizes of the T-RF's in base pairs.

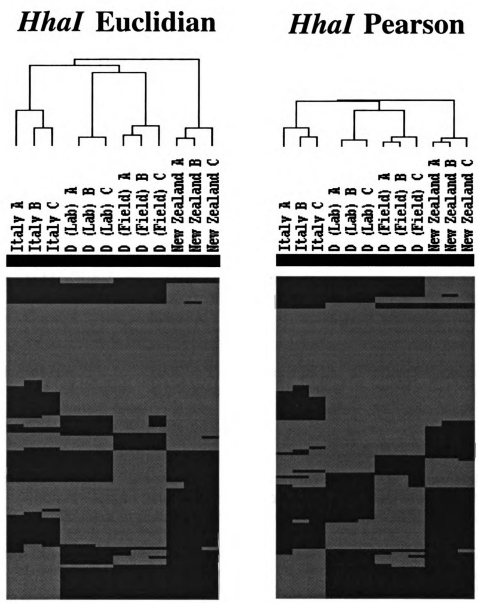

Figure 4.3: Cluster analysis of T-RFLP data obtained with *Hhal* from soil bacterial communities based on presence/absence of fragments. The dendogram on the left was calculated using Euclidian distance, the dendogram on the right with Pearson distance. Each soil was divided into three treatment groups: never treated with carbofuran (A), treated every two months with analytical carbofuran with treatments discontinuing after for six months (B-recovery), and treated continually every two months with analytical carbofuran (C).

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CHAPTER V

LINKING ACCELERATED DEGRADATION RATES OF ^{14}C-CARBONYL CARBOFURAN WITH THE PRESENCE OF THE *mcd* (CARBOFURAN HYDROLASE) GENE IN SOILS FROM AROUND THE WORLD

Abstract

Accelerated microbial degradation is an agricultural problem that arises when pesticides are repeatedly applied to the same soil resulting in reduced efficacy or failure of the compound. The objective of this study was to determine the degradation rates of ^{14}C-carbonyl carbofuran in 28 soils from around the world with different histories of carbofuran field treatment and to compare these rates to the presence or absence of the *mcd* gene, which is located on a 100kb plasmid and codes for a broad spectrum N-methylcarbamate hydrolase (carbofuran hydrolase). Degradation rates from incubation studies with moist soil and ^{14}C-carbonyl carbofuran were determined over a four week period. The occurrence of the *mcd* gene was detected by isolating community DNA and performing PCR with specific primers. The detection limit of the *mcd* gene was determined by PCR with control soils spiked with dilutions of *Aminobacter* spp. known to contain the *mcd* gene and plate counts of the dilutions. Positive signals were confirmed by sequencing the PCR product and performing a BLAST search on the results. Accelerated degradation rates were observed in 64% of the soils, and the *mcd* gene was detected in 36% of the soils. The detection limit was

identical indicating the *mcd* gene is highly conserved in different regions of the world. Of the soils where the *mcd* gene was detected, all but one showed accelerated degradation rates. The absence of the *mcd* gene in the remaining soils with accelerated degradation rates suggests that other mechanisms are responsible for the loss of carbofuran efficacy.

Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum, systemic insecticide and nematocide used to control a range of pests including corn rootworm (*Diabrotica* spp., Felsot et al. 1985), cabbage root fly (*Phorbia* spp. Karpouzas et al. 2001) and carrot fly (*Psila* spp. Suett 1986) in a variety of agricultural crops such as rice, corn and beets (Morel-Chevillet et al. 1996). Accelerated degradation of carbofuran has been found to occur worldwide (e.g. Read 1986, Turco and Konopka 1990) and is induced by repeated application to the same soil (e.g. Karpouzas et al. 2001). Previous treatments with other carbamate pesticides have also led to accelerated degradation rates of carbofuran (Harris et al. 1984, Chapman et al. 1986, Chapman and Harris 1990, Felsot 1989). As used here, the term accelerated degradation refers to the microbial metabolism at rates that are 1) higher than those determined in previously untreated soils during development and field testing of the chemicals for registration and 2) high enough to seriously affect the efficacy of the compound when applied to soil (Anderson et al. 1998).

Typical methods used to determine accelerated microbial degradation include using radioactively labeled carbofuran (e.g. Camper 1987, Charnay and Fournier 1994, Hendry and Richardson 1988) and high performance liquid chromatography (HPLC) (e.g. Ambrosoli et al. 1996, Karpouzas et al. 2001). Although these methods are very accurate, they can also be laborious and time consuming.

Nucleic acid-based technologies and the use of methyl-carbamate degrading (*mcd*) primers have the potential to more rapidly assess the genetic potential for accelerated microbial degradation of carbofuran. The *mcd* gene, which codes for carbofuran hydrolase, was cloned from *Achromobacter* spp. strain WM111 and is located on a 100kb plasmid (Tomasek and Karns 1989). This gene is highly conserved (Desaint et al. 2000, Parekh et al. 1997, Topp et al. 1993), making it useful in analyzing samples from different geographical locations.

Almost all studies with the *mcd* gene focus on pure cultures and plasmid systems (Desaint et al. 2000, Parekh et al. 1995, Parekh et al. 1997, Feng et al. 1997a, Feng et al. 1997b). To our knowledge, only one study with the *mcd* gene has been conducted on total community soil DNA (Derk et al. 1993) who found an amplification level of 33% for the *mcd* primers in 12 soils from the United States. Degradation rates of carbofuran in these soils were not reported (Derk et al. 1993). Therefore, studies correlating the presence of this gene in soil with degradation rates of carbofuran in soil are lacking.

The objective of this study was to evaluate a possible correlation between the presence of the *mcd* gene with the ability of soil to degrade carbofuran. Twenty-eight soils from around the world (five continents) with and without treatment histories of carbamate pesticides were analyzed. The implications for using this assay to make assessments of potential problem soils and as a monitoring tool will be discussed.

Materials and Methods

Pesticide

Pure analytical (chemical purity > 99%) and radiochemical (radiochemical purity > 99%) carbofuran (2,2-dimethyl-2,3-dihydrobenzo-furanyl-7-methylcarbamate) were supplied by Bayer CropScience (Monheim, Germany). ^{14}C-carbonyl carbofuran from stock solution (specific activity = 4.03 MBq/mg) was mixed with 0.12 g technical material to yield a treating solution with a final specific activity of 0.833 KBq/mg. The ^{14}C-carbonyl carbofuran treating solution was made with acetone and had a concentration of 600 µg active ingredient per 50 µl.

Soils

Twenty-eight soil samples from around the world with and without field treatment histories of carbamate pesticides were shipped to the Bayer Research Center in Monheim, Germany. The country of origin and other soil characteristics are presented in Table 5.1. Soils from the same country were taken from different

geographical locations. Each sample was a random, mixed sample from at least 10 representative sites on each field. Moisture conditions were not altered prior to shipping via air mail in plastic bags to prevent drying. Upon arrival in the laboratory, each soil was passed through a sterile, 2 mm sieve. Soils were stored in aerated containers in the dark at 4° C prior to the current study.

Soil treatments

Each soil sample was divided into two equal groups: one half was enhanced with analytical carbofuran, the other was not treated. To enhance the soils, quartz sand was used to formulate technical carbofuran dissolved in acetone (10 g quartz sand / kg dry wt. soil). After the acetone had evaporated, the formulated quartz sand was mixed with moist soil to give a final concentration of 4 mg a.i. / kg dry wt of moist soil. The untreated group received an equal amount of quartz sand treated with acetone only. The soils were incubated in the dark at 20° C for 2 months, at which time this process was repeated.

To test the degradative ability of the soils, studies with ^{14}C-carbonyl carbofuran were conducted. For this, quartz sand was formulated with ^{14}C-carbonyl carbofuran dissolved in acetone (10 g quartz sand / kg dry wt. soil) and the acetone was allowed to evaporate. Portions of 20 g dry weight soil were treated with the formulated quartz sand. After thoroughly mixing, two portions of 10 g dry wt. soil were removed and placed in 20 ml scintillation bottles. Each soil sample had a concentration of 30 mg a.i./ kg dry wt of moist soil. The water content was

adjusted to approximately 40 % water holding capacity for all samples. Each scintillation bottle with soil was placed in a separate 250 ml brown glass jar along with an additional 20 ml scintillation bottle containing 5 ml 1 N NaOH. The brown jars were sealed tightly with lids and incubated at 20° C in the dark. NaOH was replaced weekly, thereby also aerating the bottles. To determine $^{14}CO_2$ evolution, the removed NaOH was mixed with 7 ml scintillation cocktail (Quickszint 401, Zinsser Analytic, Berkshire, UK), and the amount of radioactivity determined by liquid scintillation counting (LSC) for 10 min in a LKB-Wallac 1219 Spectral liquid scintillation analyzer (Perkin Elmer Wallac GmbH, Freiburg, Germany).

DNA extraction

Total soil DNA was extracted directly from 0.3 g dry wt. soil using the FastDNA® SPIN kit for Soil (BIO101, Carlsbad, CA, USA) according to the manufacturer's instructions, except that only 700 µl (50%) of the supernatant containing DNA was added to the binding matrix for extraction, and the DNA was eluted in 100 µl DES water (BIO101). DNA from the bacterial culture was extracted with the same kit using the following modifications. The bacterial culture (2 ml) was removed from a liquid culture (described below) and centrifuged at 14000 xg for 1 min. The pellet was resuspended in 1 ml DES water and centrifuged again at 14000 xg for 1 min. The washed pellet was resuspended in 125 µl DES water which was then added to the bead column supplied in the kit. One half of the DNA suspension was added to the binding matrix for subsequent extraction

steps. The bacterial DNA was also eluted in 100 µl DES water (BIO101). The DNA concentration for the soils and bacterial culture was measured using a spectrophotometer (Perkin-Elmer, 550s UV/VIS) and a wavelength of 260 nm.

Polymerase chain reaction and gel electrophoresis

Soil and bacterial DNA were amplified in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Universal primers specific to the prokaryotic 16S rRNA, 8F Degenerate and 1392R2 (Amann et al., 1995 and Lane 1991), and a primer set for the *mcd* gene (Parekh et al. 1997) were obtained from Metabion (Martinsried, Germany).

Each 50 µl 16S rRNA PCR reaction contained 1 µl DNA template, 1x *Taq* polymerase buffer (500 mM KCl and 100 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 0.25 mM (each) of deoxynucleoside triphosphates (dTTP, dGTP, dCTP and dATP), 1 mM of each primer, 2.5 U of *Taq* polymerase (Epicentre, Madison, WI, USA). Negative control reactions did not contain template. The PCR conditions were: a pseudo hot start at 95° C for 5 min; 30 cycles consisting of 95° C for 1 min, 57° C for 1 min and 72° C for 3 min; 72° C for 7 min; a final step of 4° C forever.

Each 50 µl PCR reaction with the *mcd* primers contained 1 µl DNA template, 1x *Taq* polymerase buffer (500 mM KCl and 100 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 0.25 mM (each) of deoxynucleoside triphosphates (dTTP, dGTP, dCTP

and dATP), 1 mM of each primer, 2.5 U of *Taq* polymerase (Epicentre). Positive control reactions contained C4 (described below) DNA and negative control reactions did not contain template. The PCR conditions were: a pseudo hot start at 94° C for 3 min; 40 cycles consisting of 94° C for 20 s and 72° C for 40 s; 72° C for 3 min; a final step of 4° C forever. Amplification products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide and a 1 kb ladder size standard (Invitrogen GmbH, Karlsruhe, Germany). The *mcd* PCR product is 590 bp (Parekh et al. 1997). PCR products were purified using the QIAquick PCR cleanup kit (QIAGEN GmbH, Hilden, Germany) and sequenced on an automated sequencer using gene specific primers and the dye terminator method to confirm the presence of the mcd gene. All sequences were identified by conducting a BLAST search (www.ncbi.nlm.nih.gov) and aligned using the Staden Sequence Analysis Package (Staden 1996).

Bacterial culture

A bacterial strain (C4) known to contain the *mcd* gene was supplied by Fournier (Parekh et al. 1995). Liquid cultures of this strain were used to determine the growth curve and the degradative capacity of ^{14}C-carbonyl carbofuran. For this, 200 µg ^{14}C-carbonyl carbofuran dissolved in 0.0167 ml acetone was transferred to separate 100 ml glass jars and the acetone was allowed to evaporate. Twenty milliliters of medium containing (g l^{-1} distilled water): NH_4NO_3, 1; K_2HPO_4, 1; KH_2PO_4, 1; yeast extract (Merck, Darmstadt, Germany), 1g; $MgSO_4$ x 7 H_2O, 0.02; $Fe_2(SO_4)_3$, 0.02; and $CaCl_2$ x 2 H_2O, 0.01 was transferred to each jar

followed by 0.25 ml of the original culture. Three milliliters of 1 M NaOH was transferred to modified test tubes, i.e. holes on the side to allow gas exchange and with stoppers, and hung on the rim of each bottle. Control samples were not inoculated. Samples were incubated at 25° C in the dark and shaken at 140 rpm. At appropriate intervals over 3 days, 1 ml of culture was removed and its growth measured as a function of density by a spectrophotometer at wavelength of 600 nm. Simultaneously, the NaOH was exchanged, and the removed NaOH was mixed with 7 ml scintillation cocktail (Zinsser Analytic, Quickszint 401). The amount of radioactivity was determined by liquid scintillation counting (LSC) for 10 min in a LKB-Wallac 1219 Spectral liquid scintillation analyzer.

Cells were extracted as described above and PCR with the *mcd* primers was conducted to confirm the presence of this gene. The 16S rRNA gene was also amplified by PCR and sequenced to identify strain C4. All products were confirmed by sequencing (Qiagen GmbH, Hilden, Germany). Sequences were identified by conducting a BLAST search (www.ncbi.nlm.nih.gov).

Detection limit

Serial tenfold dilutions to the point of extinction of the culture were made in a 0.9% NaCl solution during the logarithmic growth phase. Five replicates each of the 10^{-4}-10^{-8} dilutions were plated onto 30% agar (Merck, Darmstadt, Germany) containing the medium described above and 6 mg l^{-1} carbofuran. Colonies on plates were enumerated after 2-5 days of incubation at 25° C.

In a parallel experiment, 1 ml of each dilution was removed 10 times and placed in 2 ml tubes. The samples were centrifuged at 14000 xg for 5 min and 900 µl of the supernatant was removed. The samples were then frozen at –20° C until DNA extraction in the subsequent days.

Two soils not retreated with technical carbofuran and not found to contain the *mcd* gene were used in spiking experiments to determine the detection limit. These soils were also chosen because they were on either the low or the high end of the range for percent organic carbon which can affect sorption of microorganisms and hence extraction efficiency (humic acid content).

Five samples of each soil, each 0.3 g dry wt. soil, were spiked with five separate thawed samples for each dilution and DNA was extracted as described above. PCR using the *mcd* primers was performed to determine the point at which signals ceased. 16S rRNA PCR was performed on each sample to confirm amplification of genes from these extracts was possible generally.

Nucleotide sequence accession numbers

The nucleotide sequences for fragments have been deposited in the GenBank database.

Results and Discussion

Soil degradation rates

Eighteen (64%) of the soils enhanced with technical carbofuran showed accelerated degradation rates (Table 5.2). All of these except soil 20 had a field history of carbamate pesticide use (Table 5.1). The portion of soil 20 not enhanced with technical carbofuran did not show accelerated degradation. Reports of soils with and without a field history of carbamate use showing accelerated degradation rates of carbofuran after one or two treatments in the laboratory are common (e.g. Talebi and Walker 1993, Suett and Jukes 1990).

Soils with a history of carbamate use that do not show accelerated degradation rates may contain metabolic inhibitors (Talebi and Walker 1994). For example, the organophosphorus insecticides paraoxon and disulfoton have been shown to reduce rates of carbofuran degradation by 80% (Talebi and Walker 1994). History of organophosphate use in these soils is unknown. Microbial populations capable of degrading carbofuran may also not have been present in these soils.

Nine (32%) of the soils not treated with technical carbofuran showed accelerated degradation rates. Degradation rates of the enhanced portion of these soils were considerably higher. All nine of these soils had a history of carbamate pesticide treatment, except for possibly soils 26 and 27 for which no background information is available. This history could have contributed to the accelerated rates.

PCR with *mcd* specific primers

In 36% (10) of the soils enhanced with technical carbofuran *mcd* specific PCR was successful (Table 5.2). Results did not change using lower annealing temperatures ranging from 62° C to 72° C. Of these 10 soils, all but one, soil 12, showed accelerated degradation rates. None of the soils in the untreated group showed evidence of containing the *mcd* gene. PCR amplification of total community soil DNA using 16S rRNA specific primers confirmed that DNA was extracted from all soils in both treatment groups (data not shown) and that PCR amplification was possible generally.

The 36% amplification level correlates well with a previous study of 12 soils from the United States repeatedly amended with carbofuran which reported an amplification level of 33% for the *mcd* primers (Derk et al. 1993). Amplification of the *mcd* gene in a study with 128 carbofuran-degrading bacteria was 45% (Desaint et al. 2000). In another study with bacteria, 22 of 55 carbofuran-degrading bacteria (40%) were found to have sequence homology to the *mcd* gene (Parekh et al. 1995). These results are similar to those obtained with 2,4-D degrading isolates. In 47 2,4-D degrading bacteria isolated from soils with and without a treatment history of 2,4-D, 32% exhibited sequence homology with a *tfd* gene probe (12 had homology with probes *tfd(A,B,C&D)* and three with homology to just *tfdA* (Ka et al. 1994a).

Since accelerated degradation rates of carbofuran have been found to correlate with increases in total bacterial populations (Edwards et al. 1992, Trabue et al. 2001), we expected to see positive signals in only the soils enhanced with technical carbofuran because more organisms means the relative portion of degraders increases. In a hybridization study with 2,4-D, an increase in the population of 2,4-D degraders correlated with an increase in hybridization of the soil bacterial community DNA to the *tfdA* and *tfdB* probes in response to 2,4-D addition (Holben et al. 1992).

The lack of PCR amplification with *mcd* specific primers in the rest of the enhanced soils with accelerated degradation rates implies other mechanisms of carbofuran degradation must exist. Organisms with other catabolic pathways would show no homology to the *mcd* primers and would go undetected. In a 2,4-D hybridization study an important population of 2,4-D degraders was not detected when using the *tfdA* probe in PCR analyses with total bacterial soil DNA (Ka et al. 1994b). This population was only detected with a *Spa* probe derived from 2,4-D degrading isolate *Sphingomonas paucimobilis* (Ka et al. 1994b).

Concerning soil 12, which showed amplification with the mcd primer but no evidence of accelerated degradation, other studies have reported hybridization to gene probes in soils and in bacteria that were not able to degrade the compounds. In a study with the nematocide cis-1,3-dichloropropene, PCR products from total community DNA from soil for five out of eight enhanced soils

and three unadapted soils showed sequence homology to the *dhlA* probe which encodes for the dehalogenating enzyme haloalkane dehalogenase (Verhagen et al. 1995). Seventy-six (37%) bacterial isolates that did not degrade 2,4-D were found to contain the *tfdA* gene coding for 2,4-D degradation (Hogan et al. 1997). Explanations for these false positives may include naturally occurring compounds that resemble the pesticide which serve as substrates for these organisms, or that other traits select for the occurrence of these strains. Moreover, the positive *mcd* signal for soil 12 may be from a population of microorganisms that have *mcd*-like genes that have other cell functions unrelated to carbofuran metabolism and whose population increased with carbofuran amendment. This rationale is supported by Xia et al. (1995) who showed that total community DNA from soils without a history of 2,4-D treatment hybridized to *tfdA* probes. Adding 2,4-D to previously untreated soils did not change the concentration of target *tfdA* sequence which suggests that this gene has other functions (Xia et al. 1995).

Sequence homology

Out of 485 comparable nucleotide positions, seven sequences for soils 4 (Philippenes), 10 (Costa Rica),11 (Costa Rica),12 (Costa Rica), 18 (Philippenes), 23 (South Africa) and 27 (United States) were identical (Table 5.3). Sequences for soils 22 (South Africa) and 24 (South Africa) were identical to each other and differed from the first group at two nucleotide positions (Table 3). The sequence from soil 7 (Costa Rica) was degenerate at those same two positions, indicating

that both gene species were present in, and amplified from, those soil samples (Table 5.3).

This high homology in soil DNA supports studies with isolates indicating that the *mcd* gene is highly conserved. Restriction of the amplified *mcd* gene product from 58 diverse carbofuran degrading bacteria with *AluI* or *HaeIII* endonucleases resulted in identical patterns (Desaint et al. 2000). Additional studies also report the 590 bp fragment of the *mcd* gene is highly conserved within geographically, genetically and phenotypically diverse soil bacteria (Topp et al. 1993, Parekh et al. 1997). Therefore, sequence divergence probably does not account for the lack of amplification in the other soils tested.

The presence of homologous sequences among catabolic plasmids from diverse bacteria and geographically distant soils suggests that the carbofuran degrading ability did not evolve independently in these organisms, but rather that a common set of genes, carried on a plasmid, was acquired by them (Kams 1990). Moreover, some plasmids have the ability to spread to different species at high frequencies, resulting in the rapid spread of the ability to degrade carbofuran (Head et al. 1989).

Detection limit

16S rRNA sequence information showed strain C4 to be *Aminobacter* spp., a member of the α-Proteobacteria. Sequence comparison of the *mcd* PCR product from this isolate showed sequence identity to *Achromobacter* spp. strain WM111, the only sequence for the *mcd* gene entered in the database. Approximately 41 h after inoculation (OD_{600} = 0.205), cells were harvested for serial dilutions (Figure 5.1). At this time, the *Aminobacter* sp. culture had degraded 86.9 ± 9.08% of applied carbofuran (data not shown).

PCR analyses of DNA from the spiked soils yielded identical results for both soils suggesting soil type did not affect extraction efficiency. The 10^{-6} and more concentrated dilutions consistently tested positive for five parallels, the 10^{-7} dilution tested positive for either one or two parallels, and the 10^{-8} dilution consistently yielded no amplicons (data not shown). Negative control reactions with no added template never produced PCR products, indicating that the reactions were not contaminated and that no targets were present in unspiked soils.

Plate counts of the 10^{-6} dilution of the culture averaged 620 CFU ml^{-1}. Therefore, the detection limit calculated using the plate counts and PCR was approximately ≥ 30 introduced cells for the soil sample extracted. This limit is reasonable compared to other studies: 4 to 8 x 10^2 cells/ml (Kikuchi et al. 2001), 10^2 cells/ g dry wt. soil (Rosado 1996), and 10^2 to 10^3 cells/ml (Morrow and Smets 2000).

Conclusion

In conclusion, no strong correlation exists between accelerated degradation rates of carbofuran and detection of the *mcd* gene. No clear correlation was also found for eight soils with enhanced degradation of cis-1,3-dichloropropene and the detection of the *dhlA* gene (Verhagen et al. 1995). Therefore, the *mcd* assay is most useful for monitoring the development of accelerated microbial degradation of carbofuran in soils where the majority of the degraders contain genes homologous to the *mcd* gene (Parekh et al. 1995).

The *mcd* assay can still be used to make a quick initial assessment of problem soils and is relatively inexpensive. For problem soils that show a positive signal, recommendations can be made to stop further treatments with carbofuran and other carbamate pesticides due to cross degradation. Previous treatments with carbofuran have led to increased degradation rates of other N-methylcarbamate compounds such as carbaryl, cloethocarb, propoxur, bendiocarb and trimethacarb as well as some oximic carbamates such as aldicarb, methomyl and oxamyl (Harris et al. 1984, Chapman et al. 1986, Chapman and Harris 1990, Felsot 1989).

Recently, a gene encoding for carbaryl hydrolase (*cehA*) was cloned from *Rhizobium* sp. strain AC100 which is capable of degrading carbaryl (1-naphthyl-N-methylcarbamate (Hashimoto et al. 2002). *CehA* is located in a transposon on

a plasmid, and the nucleotide sequence of the 10-kb region containing the gene has been determined (Hashimoto et al. 2002). No significant homology in the nucleotide and amino acid sequences exists between the *cehA* and *mcd* genes, indicating they are not evolutionarily related (Hashimoto et al. 2002). Future studies using both of these probes to monitor changes in microbial community structure in response to pesticide application would complement classical methods and provide a relatively unbiased assessment of the biodegradative capacity of soil.

Soil	Country	Field Carbamate History	% H_2O	$pH_{(KCl)}$	% total N	% organic C
1	Philippines	Carbofuran 1996	16.30	4.00	0.11	0.70
2	Philippines	Carbofuran 1996	14.60	4.50	0.11	0.85
3	Philippines	Carbofuran 1996	15.20	4.30	0.13	0.75
4	Philippines	Carbofuran 1996	16.00	5.60	0.13	0.69
5	Philippines	Carbofuran 1996	17.20	6.50	0.20	1.35
6	Costa Rica	Oxamyl 1995 & 1996, Carbofuran 1997	29.60	4.20	0.37	3.05
7	Costa Rica	Oxamyl 1995 & 1996, Carbofuran 1997	30.70	4.30	0.40	3.25
8	Costa Rica	Oxamyl 1996 & 1997	26.00	4.10	0.41	3.40
9	Costa Rica	Oxamyl 1996 & 1997	26.60	3.90	0.35	2.90
10	Costa Rica	No information available	28.50	4.00	0.29	2.05
11	Costa Rica	No information available	29.20	3.80	0.29	2.10
12	Costa Rica	No information available	25.90	3.70	0.28	1.40
13	Costa Rica	Oxamyl 1998	27.70	3.90	0.43	3.30
14	Costa Rica	Oxamyl 1998	31.80	4.00	0.40	3.30
15	Costa Rica	Oxamyl 1998	31.70	4.10	0.37	3.30
16	Costa Rica	Oxamyl 1998	31.10	4.20	0.43	3.75
17	Costa Rica	Oxamyl 1998	27.30	4.00	0.30	2.55
18	Philippines	Carbofuran 1996 & 1998	23.90	5.30	0.11	0.55
19	Philippines	Carbofuran 1996, 1997 & 1998,	22.70	6.30	0.12	0.95
20	Ivory Coast	None	15.60	6.80	0.18	1.40
21	Ivory Coast	Oxamyl 1998	22.60	3.70	0.56	4.05
22	South Africa	Carbofuran 1999	6.80	4.90	0.04	0.30
23	South Africa	Carbofuran 1996, Aldicarb 1994 & 1996	7.40	6.70	0.07	0.55
24	South Africa	Aldicarb 1990,1991, 1993 & 1997	5.50	6.90	0.08	0.58
25	Philippines	No information available	25.60	3.60	0.19	1.00
26	Brazil	No information available	25.80	5.90	0.33	3.49
27	United States	No information available	11.40	7.20	0.11	1.22
28	Germany	None	11.50	5.10	0.12	1.40

Table 5.1: Background information of soils used in this study. Soils from the same country were taken from separate, geographically distant locations.

soil	*mcd* presence	Cumulative % Applied ^{14}C-Carbofuran Degraded to $^{14}CO_2$ Over 4 Weeks							
		Not Treated				Treated			
		Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
1	-	0.3 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	5.6 ± 0.1	11.3 ± 0.3	17.0 ± 0.3	23.0 ± 0.2
2	-	4.0 ± 0.2	9.1 ± 0.0	16.6 ± 0.9	25.5 ± 2.4	73.6 ± 9.5	90.0 ± 13.3	93.8 ± 14.6	95.7 ± 15.5
3	-	3.2 ± 0.0	16.4 ± 0.4	34.0 ± 1.6	50.3 ± 2.9	82.4 ± 2.0	85.9 ± 3.2	87.6 ± 3.3	89.0 ± 3.2
4	yes	5.0 ± 0.1	16.0 ± 0.8	30.6 ± 0.0	44.4 ± 1.5	82.3 ± 9.9	83.1 ± 9.5	83.6 ± 9.4	84.3 ± 9.9
5	-	1.5 ± 0.0	3.1 ± 0.0	4.8 ± 0.1	6.5 ± 0.1	83.7 ± 17.8	86.9 ± 18.4	88.2 ± 18.4	89.2 ± 18.5
6	-	0.3 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.5 ± 0.0	0.9 ± 0.0	1.3 ± 0.0	1.8 ± 0.0
7	yes	0.3 ± 0.0	0.6 ± 0.0	1.0 ± 0.1	1.3 ± 0.0	6.0 ± 1.1	12.1 ± 2.4	18.5 ± 3.7	23.2 ± 2.3
8	-	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.8 ± 0.1
9	-	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
10	yes	0.4 ± 0.0	0.7 ± 0.0	1.0 ± 0.0	1.4 ± 0.0	9.7 ± 2.0	18.2 ± 5.6	27.8 ± 8.4	35.6 ± 7.3
11	yes	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	38.6 ± 0.4	56.6 ± 0.9	63.9 ± 5.4	68.3 ± 9.4
12	yes	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.3 ± 0.2	4.9 ± 0.5	7.5 ± 0.7	9.4 ± 0.5
13	-	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.2 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.8 ± 0.2
14	-	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.2 ± 0.0
15	-	0.5 ± 0.0	0.9 ± 0.0	1.5 ± 0.0	2.9 ± 0.1	0.4 ± 0.0	1.0 ± 0.0	2.0 ± 0.1	4.3 ± 0.5
16	-	7.7 ± 0.3	15.2 ± 0.6	22.6 ± 1.2	29.4 ± 3.5	76.1 ± 2.5	84.7 ± 3.6	87.0 ± 3.7	88.0 ± 3.7
17	-	0.4 ± 0.0	0.9 ± 0.0	1.9 ± 0.3	3.9 ± 2.2	0.6 ± 0.0	2.2 ± 0.2	7.2 ± 1.0	17.6 ± 2.9
18	yes	2.8 ± 0.2	31.7 ± 5.0	61.5 ± 5.1	66.4 ± 5.3	89.2 ± 3.3	90.3 ± 3.6	90.7 ± 3.6	90.8 ± 3.6
19	-	3.2 ± 0.4	6.7 ± 0.9	10.3 ± 1.5	14.2 ± 2.2	77.0 ± 3.2	88.5 ± 6.3	90.1 ± 6.6	90.8 ± 6.7
20	-	1.3 ± 0.0	2.8 ± 0.2	4.4 ± 0.4	6.2 ± 0.5	11.7 ± 0.9	45.5 ± 8.9	76.6 ± 6.8	83.3 ± 0.2
21	-	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0
22	yes	0.5 ± 0.0	1.0 ± 0.0	1.5 ± 0.1	2.0 ± 0.1	49.3 ± 0.8	71.3 ± 1.5	75.1 ± 4.8	76.5 ± 5.9
23	yes	6.6 ± 0.2	55.6 ± 0.4	78.2 ± 1.5	85.4 ± 2.4	95.5 ± 3.4	97.2 ± 3.7	97.4 ± 3.8	97.4 ± 3.8
24	yes	2.7 ± 0.1	5.3 ± 0.2	7.9 ± 0.2	10.5 ± 0.4	73.1 ± 0.7	79.7 ± 0.7	83.5 ± 1.0	86.1 ± 0.9
25	-	4.7 ± 0.1	9.1 ± 0.3	15.3 ± 1.0	26.2 ± 3.4	57.6 ± 1.5	75.8 ± 3.7	82.5 ± 4.7	85.8 ± 5.4
26	-	38.3 ± 0.4	82.6 ± 1.6	90.5 ± 1.9	93.2 ± 2.1	87.3 ± 1.4	93.6 ± 4.6	94.8 ± 5.4	95.2 ± 5.5
27	yes	13.4 ± 0.3	49.7 ± 0.7	68.6 ± 1.9	75.5 ± 2.8	82.0 ± 3.7	86.3 ± 4.1	87.9 ± 4.3	88.7 ± 4.9
28	-	2.2 ± 0.0	4.7 ± 0.0	7.2 ± 0.1	9.6 ± 0.2	1.9 ± 0.0	4.1 ± 0.0	6.3 ± 0.1	8.5 ± 0.1

Table 5.2: Results of PCR with *mcd* specific primers and degradation rates of untreated and treated soil samples. Degradation values are the averages of two replicate samples and represent the cumulative percent of applied ^{14}C-Carbofuran degraded to $^{14}CO_2$ over four weeks. Standard deviations of degradation values are also given.

Soil	Country	Sequence
4	Philippines	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
18	Philippines	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
10	Costa Rica	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
11	Costa Rica	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
12	Costa Rica	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
23	South Africa	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
27	United States	ATCAGGCTCGCTGA...GGGCTCAAGATCTAT
22	South Africa	ACCAGGCTCGCTGA...GGGCTCAAGGTCTAT
24	South Africa	ACCAGGCTCGCTGA...GGGCTCAAGGTCTAT
7	Costa Rica	AYCAGGCTCGCTGA...GGGCTCAAGRTCTAT

Table 5.3: Sequence homology of *mcd* sequences from soil samples over 485 comparable nucleotide positions.

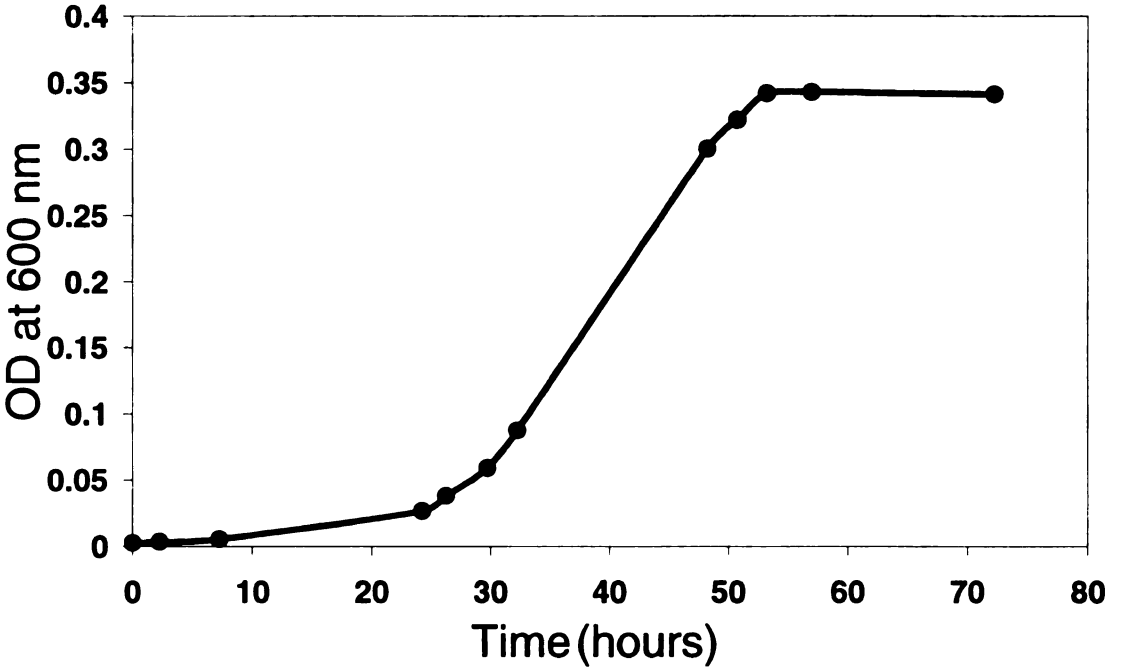

Figure 5.1: Growth curve for *Aminobacter* spp. At approximately 41 h after inoculation (OD_{600} = 0.205) cells were harvested for dilution experiments.

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